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Neurosteroids
endogenous analgesics?

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NEUROSTEROIDS; ENDOGENOUS ANALGESICS?

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MBChB, MSc, FRCA, FCARCSI

A thesis submitted in candidature for the degree of Doctor of Philosophy, University of Dundee, December 2012.

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Abbreviations

3α-HSD	3α-Hydroxysteroid dehydrogenase
5α-R	5α-Reductase
5α-THDOC	Tetrahydrodeoxycorticosterone
Allo	Allopregnanolone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	Analysis of variance
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BDZs	Benzodiazepines
Cl^-	Chloride ion
CD	Cyclodextrin
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX	Cyclo-oxygenase
Ctrl	Control
CYP450scc	Cytochrome P450 side-chain cleavage
DH	Dorsal horn
DHP	Dihydroxyprogesterone
DMSO	Dimethylsulfoxide
ECS	Extracellular solution
Finast	Finasteride
fMRI	Functional magnetic resonance imaging
$GABA_AR$	Gamma aminobutyric acid type A receptor
Ganax	Ganaxolone

GAT	GABA transporter
GlyR	Glycine receptor
HEK293	Human embryonic kidney (293) cells
ICS	Intracellular solution
Indo	Indometacin
IPSC	Inhibitory postsynaptic current
KCC2	Potassium-chloride co-transporter 2
L2/3	Neurones from layer 2/3 of the cerebral cortex
LGIC	Ligand gated ion channel
LII	Lamina II neurones of the spinal cord
mGluR	Metabotropic glutamate receptor
mIPSC	Miniature inhibitory postsynaptic current
NMDA	N-methyl-D-aspartate receptor
NSAIDs	Non-steroidal anti-inflammatory drugs
nRT	Nucleus reticularis neurones of the prethalamus
P60-75	Mice that have a (postnatal) age of 60-75 days
PAG	Periaqueductal grey
PET	Positron emission tomography
PB	Parabrachial area
SD	Standard deviation
SSI	Somatosensory cortex I
StAR	Steroidogenic acute regulatory protein (mitochondrial membrane)
T_{70}	Time taken for synaptic event to decay by 70%
τ_W	Weighted tau (exponential decay time constant)
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus

TLA	Three letter abbreviaton
TM1	Transmembrane domain 1 ($GABA_AR$)
TNF-α	Tumour necrosis factor alpha
TRPM8	Transient receptor potential subfamily M8
TRPV1	Transient receptor potential subfamily V1 (vanilloid receptor)
TSPO	Translocator protein (mitochondrial membrane)
TTX	Tetrodotoxin
VB	Ventrobasal thalamus
Veh	Vehicle
vF	von Frey filament
WDR	Wide dynamic range
WT	Wild type mouse

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I would like to acknowledge the generous support that the Wellcome Trust has provided for me to undertake a PhD looking at the underlying mechanisms responsible for neuropathy and neuropathic pain. I have taken the responsibility of my project very seriously and worked extremely hard to attempt to create a body of work that is in some way worthy of the amount of resources made available for my personal project. Over the last three years I have seen success and failure and have learned a lot about scientific research and also about human nature through my choices and mistakes. First impressions can be deceptive and the technical parts of my project that should have been the hardest challenges were all relatively straightforward. In contrast, the non-technical issues were the biggest obstacles to overcome and were typically kafkaesque in nature.

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Candidate's declaration

I declare that I am the author of this thesis, and that it is a true record of the work performed by me. This thesis has not been previously submitted for application for a higher degree. All references used in the preparation of this thesis have been consulted and are cited correctly. This work was carried out in the Centre for Neuroscience and the Institute of Academic Anaesthesia, University of Dundee, under the combined supervision of Prof Jeremy Lambert, Dr Delia Belelli and Prof Timothy Hales.

Stephen Humble

Supervisor's declaration

I certify that Stephen R. Humble has completed nine terms of experimental research and he has fulfilled the conditions of Ordinance 39, University of Dundee, such that he is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Prof Jeremy Lambert, Dr Delia Belelli and Prof Timothy Hales.

Abstract

Peripheral sensitisation and central sensitisation are implicated in the development of neuropathic pain with neuroplasticity occurring at multiple levels of the pain pathway. Hypersensitivity of the spinothalamic tract has been described in neuropathic animal models of diabetes. Spinal dorsal horn neurones of diabetic rats exhibit abnormally high spontaneous firing, suggesting an imbalance between excitatory and inhibitory signals converging within this structure. GABAergic neurones within the spinal cord and thalamus are crucial for the transmission of painful stimuli to higher centres of the brain that are involved in pain perception. $GABA_A$ receptors ($GABA_ARs$) are an important target for many clinical drugs, and certain endogenous neurosteroids act as potent allosteric modulators of these receptors. A developmental change in the rate of exponential decay of GABAergic synaptic events has been observed in other types of neurones and this may be related in part to fluctuations in endogenous neurosteroid tone. The objective of this study was to investigate changes to inhibitory neurotransmission with development in three levels of the pain pathway and to explore potential mechanisms underlying diabetic neuropathy.

The whole-cell patch-clamp technique was used on slices of neural tissue. Electrophysiological recordings were obtained from wild type mice between the ages of 6 and 80 days in lamina II of the spinal cord, the nucleus reticularis (nRT) of the thalamus and the cerebral cortex. Recordings were also obtained from mice with diabetic neuropathy (*ob/ob* and *db/db*) between the ages of 60 and 80 days. Neurosteroids and their precursors were employed along with compounds that

prevented their activity at the $GABA_AR$ such as γ-cyclodextrin, which is a barrel-shaped cyclic oligosaccharide with a lipophilic interior that sequesters neurosteroids. Behavioural experiments were also performed using von Frey filaments and the tail flick test to examine mechanical and thermal nociception.

Recordings from the spinal cord, the thalamus and the cerebral cortex revealed that the decay time of miniature inhibitory postsynaptic currents are significantly reduced with development. The neurosteroids allopregnanolone and ganaxolone were significantly more effective in neurones from the older mice. In contrast, γ-cyclodextrin had significantly less effect in neurones from the older mice. In mature diabetic mice (*ob/ob* mice), the endogenous neurosteroid tone is reduced compared to control mice, but certain neurosteroid compounds have a greater effect on the $GABA_ARs$ of these diabetic mice. In addition, the diabetic mice exhibit mechanical allodynia and hyperalgesia, which is responsive to exogenously applied neurosteroids.

These results are consistent with the hypothesis that a dramatic reduction in endogenous neurosteroid tone occurs as development progresses and that this impacts on the exponential decay time of GABAergic mIPSCs within neurones of the pain pathway. The higher neurosteroid tone in the youngest mice may confer a degree of neural protection over the nervous system as it develops. The reduction of endogenous neurosteroid tone in diabetic mice may be associated with their hypersensitivity. It is possible that pregnane-derived neurosteroids may exert analgesic effects in pathological pain states by attempting to restore the physiological GABAergic inhibitory tone that is observed in immature animals.

Chapter 1. Introduction

Part 1. Pain

1.1.1 Physiological pain

Pain is 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Merskey & Bogduk, 1994). Nociceptive (or physiological) pain refers to pain due to the stimulation of specialised peripheral nerve endings by mechanical pressure, high or low temperatures, chemical irritation or other forms of tissue damage. It has a protective role, alerting the individual to the presence of a harmful situation from which they may withdraw (Caterina *et al.,* 2005; Costigan *et al.,* 2009; Woolf & Salter, 2000). Everyone knows instinctively what pain is, but the underlying mechanisms for this highly complex phenomenon are not fully understood. The 'pain pathway' may be considered as a rational construct (based on current scientific knowledge) to explain the anatomical structures and physiological processes involved in the generation of the experience of pain.

First order neurones transmit neural impulses from the periphery to the spinal cord, where they make synaptic connections with second order neurones within the dorsal horn, which relay the signal to the thalamus *via* the spinothalamic tract as depicted in Figure 1 (Costigan *et al*., 2009; D'Mello & Dickenson, 2008). The thalamus relays the impulses onto higher centres, which constitute the so-called pain matrix *e.g*. pre-frontal cortex, limbic system, somatosensory cortex, insula and anterior cingulate cortex (D'Mello & Dickenson, 2008). The pain pathway is subject to descending modulation principally within lamina II (LII) of the dorsal horn of the spinal cord (Knabl *et al*., 2008; Munro *et al*., 2009; Takazawa & MacDermott, 2010). In pathological states, such as neuropathy, this descending modulation may be impaired (Knabl *et al*., 2008, Munro *et al*., 2009; Takazawa & MacDermott, 2010).

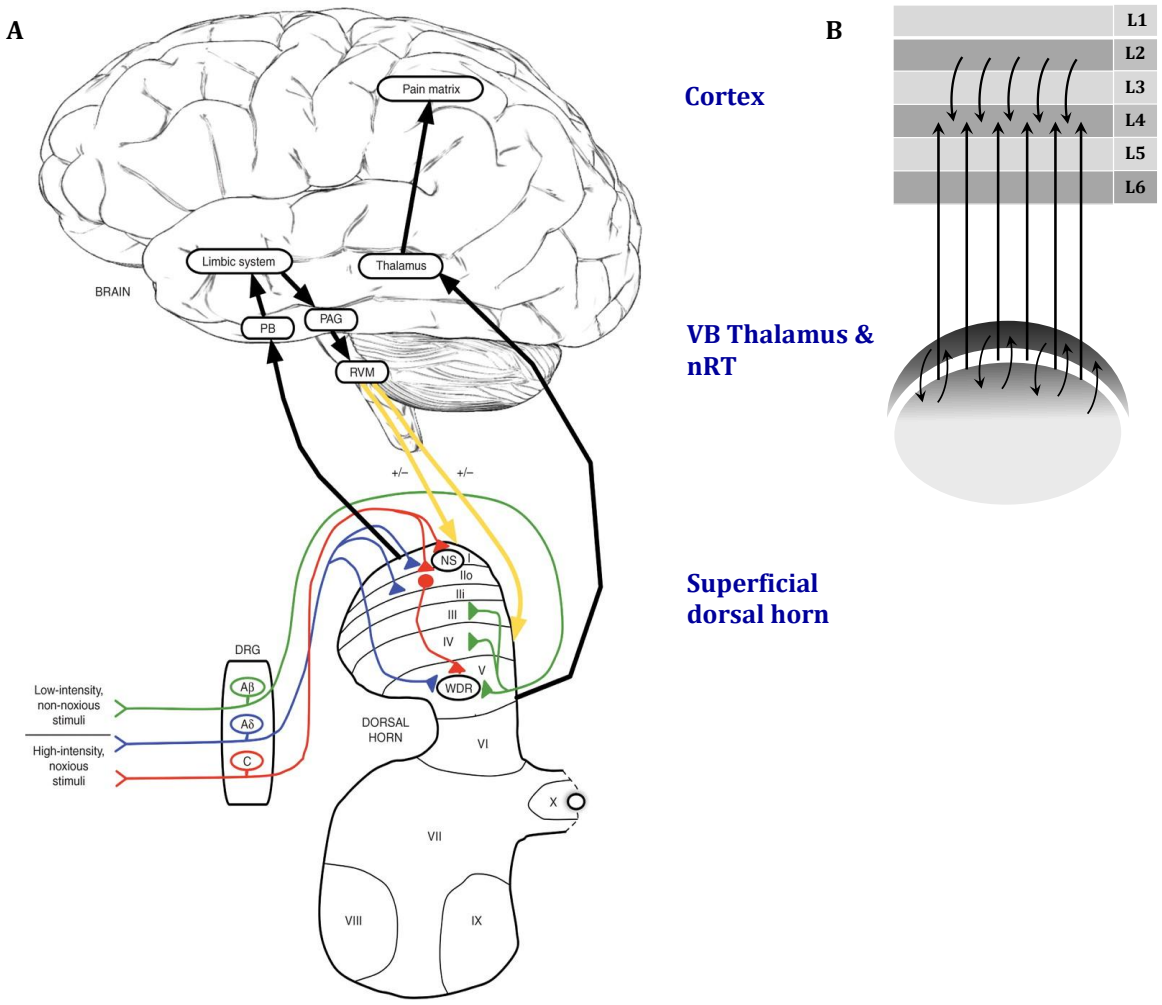

Figure 1 Pain pathway schematic.

(A) First order neurones (Aβ, Aδ and C fibres) transmit an action potential from the periphery to the dorsal horn of the spinal cord. Nociception specific neurones are principally located in laminae I and II, while wide dynamic range neurones are typically found in deeper layers such as lamina V. Second order projection neurones in the dorsal horn innervate regions such as the thalamus, the periaqueductal grey and parabrachial area. These regions relay the signals onto higher centres, which constitute the so-called pain matrix (*e.g.* pre frontal cortex, limbic system, somatosensory cortex, insula and anterior cingulate cortex). The pain pathway is subject to descending modulation by areas located within the brainstem over the superficial layers of the dorsal horn. $GABA_ARs$ and GlyRs are expressed by inhibitory neurones within the dorsal horn of the spinal cord and these mediate descending inhibitory tone over spinal second order projection neurones (Adapted from D'Mello & Dickenson, 2008). **(B)** The reciprocal inter-relationship between the VB and nRT creates a fine balance of excitation and inhibition of nociceptive transmission to the cortex. The cortex itself is organised in parallel columns (Layers 1-6) that function in modules with vertical excitatory and inhibitory loops.

1.1.2 Nociception

Specialised sensory neurones known as primary afferent nerves have peripheral cutaneous and visceral nociceptive terminals that are activated by a range of stimuli, including mechanical, thermal and chemical insults (Caterina *et al.,* 2005; Costigan *et al.,* 2009; Woolf & Salter, 2000). Direct activation of ion channel proteins such as transient receptor potential (TRP) channels on the nociceptive terminal, or stimulation of neurotrophin, or G protein-coupled receptors initiates the depolarisation of the sensory nerve fibre (Caterina *et al.,* 2005; Costigan *et al.,* 2009; Woolf & Salter, 2000). Depolarisation results in the initiation of an action potential, which is transmitted from the periphery to the spinal cord. The principle types of sensory neurones in the mammalian nervous system are Aβ, Aδ and C fibres. The Aβ fibres typically have a larger diameter and are more highly myelinated than Aδ fibres, which enables them to conduct neural impulses at a great velocity (Caterina *et al.,* 2005; D'Mello & Dickenson, 2008; Millan, 1999). C fibres lack myelin and are of even smaller diameter than Aδ fibres (0.4-1.2μM and 2-6μM respectively), thus making them the slowest conductors. Additionally, Aβ fibres have low activation thresholds, which means that they respond rapidly to innocuous stimuli such as light touch (Caterina *et al.,* 2005; D'Mello & Dickenson, 2008; Millan, 1999). In contrast, Aδ and C fibres have higher activation thresholds and respond to painful, or noxious mechanical, or thermal stimuli (Caterina *et al.,* 2005; D'Mello & Dickenson, 2008; Millan, 1999). The different properties of these first order afferent neurones facilitate the processes of sensory discrimination and the monitoring of the physical environment, which confer survival advantages for the individual. Conversely, the loss of nociception as a result of a loss of function mutation of the gene encoding for the sodium channel $Na_v1.7$ is associated with repeated and disabling self-injury (Costigan *et al.,* 2009; Cregg *et al.,* 2010).

1.1.3 Spinal cord

The mammalian spinal cord is a highly complex neurological structure that conducts, integrates and modulates neural signals between the brain and the

peripheral nervous system. A cross section through the spinal cord reveals a circumferential outer layer known as white matter, which contains sensory and motor nerve tracts (Hunt & Bester, 2005). Located within the white matter is the butterfly-shaped grey matter, which consists of nerve cell bodies. The grey matter is divided into ten layers (or Rexed's laminae I-X) according to differences in cell structure. Laminae I-VI are located within the dorsum of the spinal cord and comprise the dorsal horn. First order afferent neurones terminate within the dorsal horn, where they synapse with second order projection neurones. The superficial laminae I and II (LI & LII; also known as substantia gelatinosa) are integral to spinal nociceptive processing, while the deeper laminae V, VI and X also play a role (Chery & De Koninck 1999; D'Mello & Dickenson, 2008; Millan, 1999). Cutaneous afferent C fibres project primarily to LII while Aδ fibres more commonly project to LI. Unmyelinated afferent fibres from muscles, joints and organs typically project to LI, LV, LVI and LX. There is a substantial degree of convergence of afferent fibres from different locations onto individual spinal neurones. For example, somatosensory afferents from the left upper arm frequently share spinal pathways with visceral afferents from the heart. This organisation means that myocardial infarction may present with 'referred' left arm pain. Wide-dynamic range (WDR) neurones in particular receive multiple convergent afferent fibres from a range of tissue (D'Mello & Dickenson, 2008; Millan, 1999). WDRs are found predominantly in LIV-LVI, but also in LI, LII and LX. Their name refers to their ability to produce a variable stimulus-dependent response over a wide range of afferent input and they also mediate the 'wind-up' phenomenon (D'Mello & Dickenson, 2008; Mendell & Wall, 1965; Millan, 1999).

LI has the highest concentration of projection neurones within the dorsal horn, these neurones project to areas such as the thalamus, hypothalamus, nucleus of the tractus soliarius, periaqueductal grey (PAG) and parabrachial (PB) areas (D'Mello & Dickenson, 2008; Millan, 1999; Todd & Ribeiro da Silva, 2005). The axons of the projection neurones typically cross the midline of the spinal cord and ascend within the anterolateral region of the white matter (Todd & Ribeiro da Silva, 2005). Animal models have been used to provide important insights into the molecular mechanisms that occur in the mammalian nervous system. In rats, LI

neurones of the spinal cord are subject to fast synaptic inhibition mediated by strychnine-sensitive glycine receptors (GlyRs), while gamma-aminobutyric acid type A receptors ($GABA_ARs$) mediate extra synaptic tonic inhibition (Chery & De Koninck, 1999; Lynch, 2009; Mitchell *et al.,* 2007).

In contrast, LII neurones receive fast synaptic inhibition mediated by GlyRs and $GABA_ARs$ (Chery & De Koninck, 1999; Keller *et al.,* 2001). Pharmacological blockade of either the $GABA_AR$ or the GlyR results in hyperexcitability of dorsal horn neurones, which is associated with neuropathic pain symptoms (Charlet *et al.,* 2008; Yaksh, 1989). The organisation of LII is complex, however, there are considered to be four main cell-types, including: islet, vertical, radial and central cells (Yasaka *et al.,* 2010). Islet cells function as inhibitory interneurones, which may have projections to LI. Vertical and radial cells are usually (but not always) glutamatergic and therefore excitatory. Central cells may also be glutamatergic or GABAergic (Yasaka *et al.,* 2010). LII may also be subdivided into the inner and outer layers: LII_I and LII_O respectively. The different cell types within LII enable this layer to have a critical role in the transmission, integration and modulation of nociceptive signals within the dorsal horn (Poisbeau *et al.,* 2005). For these reasons, LII has been the focus of research seeking to examine the implications of the loss of inhibitory tone that may be associated with pathological hypersensitivity (Chen & Pan, 2002; Keller *et al.,* 2001; Millan, 1999; Poisbeau *et al.,* 2005; Todd & Ribeiro da Silva, 2005; Yaksh, 1989).

The majority of Aδ and C nerve fibres terminate centrally in the superficial layers of the dorsal horn of the spinal cord: laminae I-II, while most Aβ fibres terminate in the deeper layers: laminae III-VI (D'Mello & Dickenson, 2008; Todd & Ribeiro da Silva, 2005). Second order spinal neurones, which receive synaptic projections from the afferent first order neurones, may be classified into three principle categories: 1) Nociception specific neurones located superficially; 2) Proprioception specific neurones located in the deeper laminae and; 3) WDR neurones which receive synaptic projections from Aβ, Aδ and C fibres and are able to relay a graded response to higher centres *via* LIII-VI (D'Mello & Dickenson, 2008, Hunt & Bester, 2005; Millan, 1999).

The principle excitatory neurotransmitter in the spinal cord and the entire central nervous system (CNS) is the amino acidglutamate (Rousseaux, 2008a). Glutamate, which is released from the presynaptic nerve terminal, activates the postsynaptic receptor and thus initiates depolarisation. The major classes of ionotropic glutamate receptors are the NMDA, AMPA, and kainate receptors, and a family of G-protein coupled receptors (metabotropic glutamate receptors mGluRs; Bleakman *et al.,* 2006; Dickenson, 2011; D'Mello & Dickenson, 2008; Petrenko *et al.,* 2003; Rousseaux, 2008a; Traynelis *et al.,* 2010). Acute, or low frequency, noxious impulses to the spinal cord activate the AMPA receptors *via* the release of glutamate, while repetitive, or high frequency, impulses from C fibres activate the NMDA receptors. NMDA receptors do not conduct until the cell depolarises *via* AMPA receptor activation and the magnesium ion block is removed by prolonged depolarisation associated with substance P and other peptides (Dickenson, 2011; D'Mello & Dickenson, 2008). NMDA receptors are implicated in the induction and maintenance of central sensitisation (Bleakman *et al.,* 2006; Petrenko *et al.,* 2003; Rousseaux, 2008a; Traynelis *et al.,* 2010).

The NMDA receptor (NMDAR) is comprised of four (variable) subunits that may be assembled in different configurations (GluN1, GluN2A-D, GluN3A & GluN3B) to produce the individual NMDAR isoforms (Bleakman *et al.,* 2006; Petrenko *et al.,* 2003; Rousseaux, 2008a; Traynelis *et al.,* 2010). These isoforms are differentially distributed within the CNS (Millan, 1999; Rousseaux, 2008a). Each NMDA receptor isoform has individual pharmacological properties, but they all exhibit relatively slow channel kinetics, a variable degree of magnesium ion block and are permeable to calcium ions (Bleakman *et al.,* 2006; Millan, 1999; Petrenko *et al.,* 2003; Rousseaux, 2008a; Traynelis *et al.,* 2010). NMDA receptors also have a recognition site for glycine (or D-serine), the occupation of which is necessary for the function of the associated ion channel (Bleakman *et al.,* 2006; Millan, 1999; Rousseaux, 2008a; Traynelis *et al.,* 2010). The voltage-dependent magnesium ion 'plug' remains within the channel and inhibits receptor activation until it is removed by prolonged depolarisation of the postsynaptic membrane, aided by co-transmitters such as substance P and CGRP (Dickenson, 2011; D'Mello &

Dickenson, 2008; Rousseaux, 2008a; Traynelis *et al.,* 2010). Thus the NMDA receptor requires more noxious and prolonged stimuli for activation and results in a more amplified and sustained response of the second order neurones. This phenomenon is referred to as 'wind-up.' Indeed, the NMDA receptor is implicated in the development of hypersensitivity to pain that is observed in inflammation or neuropathy (Dickenson, 2011; D'Mello & Dickenson, 2008; Petrenko *et al.,* 2003). Activation of the NMDA receptor results in a large influx of calcium, which depolarises the membrane and triggers a cascade of secondary messengers that activate enzymes such as protein kinase C, calcium-calmodulin dependent protein kinase II and phospholipase A and C (D'Mello & Dickenson, 2008; Rousseaux, 2008a). An excitatory post synaptic potential induces sodium ion flux *via* voltage-gated sodium channels, which generates an action potential. The action potential is transmitted by the second order spinal projection neurones of the dorsal horn *via* the ascending tracts to higher areas such as the thalamus (D'Mello & Dickenson, 2008; Rousseaux, 2008a; Traynelis *et al.,* 2010).

The AMPA receptor (AMPAR) is comprised of four subunits and may be assembled as a homomeric, or heteromeric, configuration (GluA1-4; Bleakman *et al.,* 2006; Rousseaux, 2008a; Traynelis *et al.,* 2010). Calcium permeable AMPARs are located within the superficial laminae of the dorsal horn of the spinal cord and can act presynaptically to enhance the spontaneous release of GABA from GABAergic inhibitory neurones (Bleakman *et al.,* 2006; Rousseaux, 2008a; Traynelis *et al.,* 2010). AMPARs are also expressed ubiquitously in brain regions associated with nociception, including the brainstem, thalamus and sensory cortices (Bleakman *et al.,* 2006; Rousseaux, 2008a; Traynelis *et al.,* 2010). Selective AMPAR antagonists are able to suppress the firing of WDR neurones of the dorsal horn in response to both noxious and mild stimuli, suggesting that AMPARs are involved in nociceptive and sensory transmission (Bleakman *et al.,* 2006; Rousseaux, 2008a). Indeed, AMPARs may also be involved in the phenomenon of 'wind-up' (Bleakman *et al.,* 2006; Mendell & Wall, 1965; Traynelis *et al.,* 2010).

The kainate receptor has a similar tetrameric structure to the AMPAR and may be expressed in either homomeric, or heteromeric, configurations of the various

subunits (GluK1-5; Bleakman *et al.,* 2006; Rousseaux, 2008a; Traynelis *et al.,* 2010). These receptors are distributed within areas of the nociceptive pathway and have a similar role to AMPARs (Bleakman *et al.,* 2006; Rousseaux, 2008a; Traynelis *et al.,* 2010).

The metabotropic glutamate receptors (mGluR) are a family of G-protein coupled receptors that modulate the presynaptic release of glutamate and the postsynaptic sensitivity to glutamate (Rousseaux, 2008a). Multiple mGluR receptor subtypes (mGluR1-8) are expressed on pre- and post-synaptic neurones of both the ascending and descending nociceptive tracts of the spinal cord (Bleakman *et al.,* 2006; Rousseaux, 2008b). mGluR subtypes 1,5 and 6 stimulate inositol phosphate metabolism and calcium mobilisation, mGluR subtypes 2-4 and 6-8 are linked to adenylcyclase (Rousseaux, 2008a). Increases in mGluR activity within the spinal cord have been observed in inflammatory and neuropathic pain, coinciding with the manifestation of hypersensitivity (Bleakman *et al.,* 2006, Rousseaux, 2008b). mGluRs expression at higher levels of the nociceptive pathway may also be increased in neuropathic pain states (Bleakman *et al.,* 2006; Rousseaux, 2008b).

1.1.4 Descending modulation

Historically, pain pathway neurones as described above were considered to remain inert until a noxious event occurred to activate them. However, it is now understood that the nervous system is continually active in its physiological role as a monitoring system of the external environment. Graded ascending impulses are constantly being sent from the periphery, even in resting conditions and descending pathways from higher centres influence these impulses (Gebhart & Proudfit, 2005). Neurones in the ventrolateral periaqueductal grey (PAG) region of the midbrain regulate the activation of neurones in the rostroventral medulla (RVM) region and the dorsolateral pontine tegmentum (DLPT) (Gebhart & Proudfit, 2005). The RVM and DLPT both have separate descending neural pathways that project to the spinal cord. These brainstem pathways can mediate an inhibitory, or an excitatory influence over nociceptive transmission in the

dorsal horn (Gebhart & Proudfit, 2005). Thus this system can act as a regulatory mechanism for the transmission of sensory information from the peripheral nervous system to higher centres. Interestingly, electrical or pharmacological stimulation of the PAG has been shown to have an analgesic effect, while loss of physiological inhibitory tone is associated with hypersensitivity to pain (D'Mello & Dickenson, 2008; Gebhart & Proudfit, 2005). The underlying mechanisms responsible for mediating descending modulation are complex and not fully understood, but this system has been a target for analgesic or pain-relieving drugs. Opioid receptors and endogenous opioid compounds have been discovered in the brainstem and neurones descending from the RVM mediate their actions *via* serotonin receptors and adrenoceptors (Gebhart & Proudfit, 2005). These findings provided a rationale for the effectiveness of opioid drugs and tricyclic antidepressants for the management of pain and/or hypersensitivity respectively (D'Mello & Dickenson, 2008; Gebhart & Proudfit, 2005). In clinical practice, opioid drugs are very effective for acute nociceptive and inflammatory pain (Macintyre & Schug, 2007). However, both opioids and tricyclic antidepressants have limited effectiveness in chronic neuropathic pain and hypersensitivity (Dworkin *et al.,* 2007; 2010; Macintyre & Schug, 2007). Despite increasing knowledge of the underlying pathophysiology of neuropathic pain there has been relatively little change in clinical management over recent years (Dworkin *et al.,* 2007; 2010; Macintyre & Schug, 2007). Indeed, neuropathic symptoms are untreatable in many people with conditions such as diabetic neuropathy or phantom limb pain, which places a large burden on society and provides an incentive for further, detailed research of this field (Bouhassira *et al.,* 2008).

$GABA_A$Rs are the major inhibitory receptors in the mammalian nervous system (Reviewed in: Belelli & Lambert, 2005; D'Hulst *et al.,* 2009). $GABA_A$Rs and GlyRs are expressed by inhibitory neurones within the dorsal horn of the spinal cord (Chery & De Koninck, 1999; Keller *et al.,* 2001; Mitchell *et al.,* 2007). Activation of $GABA_A$Rs is allows a chloride ion flux which usually causes hyperpolarisation of the postsynaptic membrane, a reduction of the potential for depolarisation and also shunting of membrane resistance (see Part 2 of the Introduction for a detailed description of the $GABA_A$R). Hence, $GABA_A$Rs may mediate descending inhibitory

tone over spinal second order projection neurones and loss of GABAergic function is associated with hypersensitivity manifested as allodynia, or hyperalgesia (Munro *et al*., 2009; von Hehn *et al*., 2012; Yaksh, 1989; Zeilhofer, 2008).

1.1.5 Ascending tracts

Neurones from the dorsal horn of the spinal cord project rostrally to higher centres such as: the periaqueductal grey, the parabrachial area and the dorsal reticular nucleus within the brainstem, the thalamus, the limbic system and the cerebral cortex (Hunt & Bester, 2005). Signals travel *via* multiple and complex ascending nociceptive pathways that reach the supraspinal structures by direct and indirect routes (Hunt & Bester, 2005; Millan, 1999). For instance, the parabrachial nucleus is the target for several ascending pathways, however, it also sends neuronal projections up to the thalamus and back down to the spinal cord (Hunt & Bester, 2005; Millan, 1999). The parabrachial nucleus is a region of the pons involved the integration of the autonomic and motivational response to pain (Hunt & Bester, 2005; Millan, 1999). The classic three monosynaptic pathways are the spinothalamic, spinoreticular and spinomesencephalic tracts (Millan, 1999). The neurones within these tracts cross the midline to ascend on the contralateral side, while neurones belonging to polysynaptic tracts typically remain on the ipsilateral side (Hunt & Bester, 2005; Millan, 1999).

1.1.6 Thalamus

The thalamus is considered to be the key region for the relay and modulation of sensory transmission from the periphery to higher centres. It integrates discriminative information concerning the location, intensity, type and temporal pattern of the nociceptive stimulus (Clasca *et al.,* 2012; Millan, 1999). The thalamus may be divided into multiple substructures with different structural and functional characteristics that act in concert. In rodents, the ventrobasal (VB) complex is comprised of three components: ventroposteriolateral (VPL), ventroposteriomedial (VPM) and ventroposterioinferior (Clasca *et al.,* 2012; Millan, 1999). The VPL and VPM are important for the integration of sensory

information related to the sensory-discriminative components of pain such as intensity and location. In contrast, the VPI and the posteriolateral region of the thalamus are involved in the affective-cognitive components of pain such as emotional distress and escape/avoidance behaviour. The distinct areas of the thalamus tend to innervate separate regions of the cortex according to their specialist functions (Clasca *et al.,* 2012; Millan, 1999). For instance, the VPL and VPM have strong connections with the somatosensory cortex I (SSI) which is involved in the sensory-discriminative component of pain. Other parts of the thalamus such as the VPI and the posteriolateral region that are concerned with the affective-cognitive components of pain primarily innervate relevant cortical areas such as the SSII and the insula, pre-frontal, parietal and cingulated cortices (Millan, 1999). Thalamic somatosensory projection neurones from areas such as the VB arborise, or branch out principally to layers III-IV of the cerebral cortex. Other layers of the cortex also receive axonal projections from those areas of the thalamus, but to a lesser degree (Clasca *et al.,* 2012; DeFelipe & Farinas, 1992; Lubke *et al.,* 2000; Lubke & Feldmeyer, 2007; Mountcastle, 1997; Treede *et al.,* 1999).

1.1.7 nRT

In rodents, the nucleus reticularis (nRT) consists of a crescent of neural tissue that encircles the ventral surface of the oval-shaped thalamus. The nRT is part of the pre-thalamus and it is an important regulator of somatosensory transmission *via* the relay neurones of other parts of the thalamus such as the VB (Arcelli *et al.,* 1997; Cox *et al.,* 1997; Gentet & Ulrich, 2003; Guillery & Harting, 2003; Huh *et al.,* 2012). The nRT is comprised of GABAergic inhibitory neurones that receive input from pyramidal neurones of the cerebral cortex, but do not project back to the cortex directly themselves (Gentet & Ulrich, 2003; Guillery & Harting, 2003). nRT neurones are the main source of GABAergic regulation of VB neurones *via* closed disynaptic loops, which mediate reciprocal and lateral inhibition (Arcelli *et al.,* 1997; Cox *et al.,* 1997; Gentet & Ulrich, 2003; Guillery & Harting, 2003; Huh *et al.,* 2012; Figure 1). The inter-relationship between the nRT and VB creates a fine

balance of excitation and inhibition, which modulates nociceptive transmission and can therefore ultimately influence nociceptive behaviour in live animals (Guillery & Harting, 2003; Huh *et al.,* 2012). VB neurones that project to the cerebral cortex exhibit both tonic and burst firing patterns (Cheong *et al.,* 2008; 2011). The tonic and burst firing patterns are regulated by the phospholipase C β4-protein kinase C cellular transduction pathway, which modulates both T- and L-type calcium channel activity (Cheong *et al.,* 2008; 2011). The nRT regulates the predominant pattern within an individual VB neurone and can therefore increase, or decrease, sensitivity to a painful stimulus such as inflammation (Cheong *et al.,* 2008; 2011; Huh *et al.,* 2012). Specifically, PLCβ4-deficient mice have a diminished visceral pain response, which is related to an increase in burst firing with a decrease in tonic firing of thalamocortical neurones (Cheong *et al.,* 2008).

1.1.8 Cerebral cortex

Historically it was believed that there was a single pain centre somewhere within the brain that would be activated in response to a peripheral noxious stimulus (Wall *et al.,* 2006). However, pain has sensory, emotional and cognitive components and therefore multiple areas within the cerebral cortex (or neocortex) are involved in generating the experience of pain (Flor & Bushnell 2005; Treede *et al.,* 1999; Wall *et al.,* 2006). These areas may be considered as the 'pain matrix.' There have been numerous neuroimaging studies to attempt to identify the implicated regions however the results obtained have often been inconsistent. Despite the uncertainty, a number of key cortical areas have been identified: the somatosensory cortices I-II and the insula, pre-frontal, parietal and cingulated cortices (Flor & Bushnell, 2005; Millan, 1999; Moisset & Bouhassira, 2007; Treede *et al.,* 1999; Treede & Apkarian, 2007; Wall *et al.,* 2006). Other regions of the brain such as the cerebellum and the basal ganglia are also thought to play minor roles in the phenomenon of pain (Flor & Bushnell, 2005; Treede & Apkarian, 2007).

In rodents the cerebral cortex is a thin layer with a large area that overlies the rest of the brain. There are billions of cortical neurones that exist in the form of a

complex integrated network with trillions of synaptic connections to facilitate the rapid transfer of neural impulses between cells (Ben Ari & Spitzer, 2010; Mountcastle, 1997). The cerebral cortex is organised into parallel mini-columns of synaptically linked neurones that span part, or all of the six horizontal cortical layers. Multiple mini-columns may also be clustered together to constitute functional modules (Lubke *et al.,* 2003; Lubke & Feldmeyer, 2007; Meyer *et al.,* 2011; Mountcastle, 1997).

Within the cerebral cortex there are two types of neurones: spiny and aspiny. The spiny neurones may be further subdivided into pyramidal and non-pyramidal cells. Pyramidal neurones account for around 80 percent of cortical neurones and consist of a pyramid-shaped body with long axons that project into the white matter. They are the major output fibres and found in all layers except for layer 1 (DeFelipe & Farinas, 1992). Spiny non-pyramidal cells have short axons and function as interneurones within the cortical network. Cortical interneurones have heterogenous properties and have been described and classified according to their variable morphological, physiological and molecular characteristics (Ascoli *et al.,* 2008). There is a degree of heterogeneity in the electrophysiological properties of cortical neurones, but there appears to be little difference between different regions of the cerebral cortex such as the sensorimotor and the cingulate cortices (Brown, 2012; Lubke & Feldmeyer, 2007; McCormick *et al.,* 1985; Viaene *et al.,* 2011).

All cortical regions receive communications from the thalamus and other cortical areas *via* corticothalamocortical and corticocortical pathways organised in parallel (Sherman & Guillery, 2011; Viaene *et al.,* 2011). These pathways may be considered as 'drivers' and 'modulators' respectively (Sherman & Guillery, 2011; Viaene *et al.,* 2011). Trans-thalamic pathways incorporate the thalamus as a GABAergic gating system, which can be opened, or closed (Sherman & Guillery, 2011; Viaene *et al.,* 2011). Layer 4 neurones are the principle target for thalamocortical 'driver' (or afferent) inputs and they relay the neural signal to layer 2/3 *via* strong excitatory connections (Viaene *et al.,* 2011; 2011b). Layer 2/3 neurones mostly receive 'modulatory' thalamic inputs and have a more complex

involvement in neural processing and the generation of cortical output signals (Bannister, 2005; Eto *et al.,* 2011; Lubke *et al.,* 2000; Lubke & Feldmeyer, 2007; Sherman & Guillery, 2011; Viaene *et al.,* 2011; 2011b).

GABAergic inhibitory interneurones are present in all layers of the cortex but are most abundant in layers 2/3 and lower in layer 4 (Meyer *et al.,* 2011). They project vertically down to the lower layers, exerting GABAergic inhibitory tone on pyramidal cells within the column (Helmstaedter *et al.,* 2009; Mountcastle, 1997). These connections create, in effect, narrow vertical loops of excitation and inhibition, but there are also horizontal connections between neurones within layer 2/3 (Helmstaedter *et al.,* 2009). Despite the large amount of work that has focused on this area, understanding of these complex processes and their functional significance remains relatively limited (Lubke & Feldmeyer, 2007; Treede & Apkarian, 2007).

1.1.9 Inflammatory pain

Tissue damage results in the release of chemical substances referred to as inflammatory mediators. There are numerous inflammatory mediators which act *via* different mechanisms to lower the activation thresholds of peripheral nociceptors, thereby promoting peripheral sensitisation (Caterina *et al.,* 2005; Costigan *et al.,* 2009). The exact mechanisms responsible for this inflammatory sensitisation process have not yet been fully elucidated, but substances such as protons, prostaglandins, ATP, serotonin, bradykinin, BDNF, TNF-α and arachidonic acid are able to directly, or indirectly, temporarily enhance the sensitivity of receptors such TRPV1, ASIC and TRPM8 (Costigan *et al.,* 2009; Harvey & Dickenson, 2008). The consequence of sensitisation of TRPV1 is that these channels, which are normally activated by noxious, or harmful, temperatures, may be activated at body temperature and therefore result in spontaneous pain (Caterina *et al.,* 2005; Woolf & Salter, 2000). The physiological benefit of this process is the enforced resting of the injured area of the body in order to optimise tissue healing, however chronic inflammation such as osteoarthritis, or

rheumatoid arthritis, can be disabling diseases associated with perpetual hypersensitivity (Costigan *et al.,* 2009; Harvey & Dickenson, 2008).

1.1.10 Neuropathic pain

Neuropathic pain results from a dysfunctional, or damaged, nervous system and may be associated with pathological neuroplasticity within the peripheral, or central, nervous system (Harvey & Dickenson, 2008; Merskey & Bogduk, 1994, Woolf & Salter, 2000). The underlying pathophysiological processes are highly complex and not fully understood, but involve the interaction of the immune and nervous systems (Costigan *et al*., 2009; Scholz & Woolf, 2007). Neuropathic pain may be characterized by spontaneous sharp, shooting, or burning sensations associated with sensory loss and paradoxical hypersensitivity (Costigan *et al*., 2009; Kehlet *et al*., 2006). Hyperalgesia describes an exaggerated response to a painful stimulus, while allodynia describes an unpleasant, or painful, response associated with an innocuous stimulus (Merskey & Bogduk, 1994).

A number of disorders are associated with neuropathic pain, including diabetes mellitus, post-herpetic neuralgia, phantom limb pain, complex regional pain syndrome and cancer (Freynhagen & Bennett, 2009). Intractable neuropathy and neuropathic pain have a large impact on society (Bouhassira *et al*., 2008; Harstall & Ospina, 2003) and the treatment options available such as opioids often have very limited efficacy and significant side effects (Dworkin *et al*., 2007; 2010). By improving the understanding of these conditions at the molecular level it may be possible to identify novel therapeutic targets.

Both peripheral and central sensitisation are implicated in the development of painful diabetic neuropathy (Fischer & Waxman, 2010). Hypersensitivity of the spinothalamic tract has been described in neuropathic animal models of type I diabetes (Chen *et al*., 2001, Pertovaara *et al*., 2001). In particular, the spinal dorsal horn neurones of diabetic rats exhibit abnormally high rates of spontaneous action potential discharge, suggesting an imbalance between excitatory and inhibitory

signals converging within this structure (Chen & Pan 2002). These neurones are crucial to the transmission of painful stimuli to the higher centres of the brain involved in pain perception (Hunt & Bester, 2005; D'Mello & Dickenson, 2008).

1.1.11 Diabetes

Diabetes mellitus is a very common and devastating disease, which has a large socioeconomic cost (Davies *et al.,* 2006; Edwards *et al.,* 2008; Kaplan & Wagner, 2006). In type-1 diabetes mellitus (T1DM) there is a lack of insulin due to autoimmune destruction of the β cells of the islets of Langerhans in the pancreas (Mathis *et al.,* 2001). Type-2 diabetes mellitus (T2DM) is more common and is characterised by a loss of sensitivity to insulin and the eventual inability of the islets of Langerhans to compensate (Buchanan, 2003; De Fronzo, 2004). These pathological processes result in the development of hyperglycaemia and current management is focused primarily on the optimisation of blood glucose levels in order to reduce the severity of neuropathy (Edwards *et al.,* 2008). The underlying mechanisms for the development of diabetic neuropathy are not well understood but appear to involve oxidative stress and protein kinase signalling pathways in response to hyperglycaemia (Edwards *et al.,* 2008).

Individuals with diabetes typically suffer from significant macrovascular and microvascular complications including neuropathy (Boulton, 2004; Cefalu, 2006; Davies *et al.,* 2006). This condition can manifest itself in ischaemic heart disease and peripheral vascular disease, which often leads to chronic pain, or even lower limb amputation (Boulton, 2004; Davies *et al.,* 2006; Edwards *et al.,* 2008). However, these complications usually take many years to develop, which makes it challenging to assess the impact of therapeutic interventions. Therefore it is important to utilise animal models to facilitate the development of effective clinical management (Cefalu, 2006; Kaplan & Wagner, 2006; Rees & Alcolado, 2005).

1.1.12 Diabetic mouse models

In mice, diabetic neuropathy develops over weeks, which allows disease progression and the efficacy of interventions to be studied within a relatively a short timeframe (Cefalu, 2006; Kaplan & Wagner, 2006; Neubauer & Kulkarni, 2006; Rees & Alcolado, 2005). T2DM is the more common type seen in humans (90 percent of diabetics) and with the burgeoning obesity epidemic in the developed world the incidence is likely to become even greater (Buchanan 2003; Cefalu 2006; Davies *et al.,* 2006; Edwards *et al.,* 2008; Kaplan & Wagner 2006). Taking these factors into consideration, it was decided to focus on T2DM for the purposes of this project. A complete discussion of the complex pathophysiological mechanisms implicated in the development of diabetes mellitus and diabetic neuropathy is beyond the scope of this thesis, but the matter has been the subject of several in-depth reviews (Buchanan 2003; Cefalu 2006; Dobretsov *et al.,* 2007; Edwards *et al.,* 2008; Mathis *et al.,* 2001). There are multiple established mouse and rat models of diabetes, but no single animal model fully replicates the condition in humans ergo, all models have pros and cons (Cefalu, 2006; Kaplan & Wagner, 2006; Mathis *et al.,* 2001; Sullivan *et al.,* 2007).

For the purposes of this study the most important issue was to identify a model which exhibited consistent features of neuropathy and neuropathic hypersensitivity. Mouse models of diabetes are useful because they have a short life cycle, with a relatively rapid development of disease symptoms and are readily available from commercial sources (Kaplan & Wagner, 2006). In addition, with specific reference to the $GABA_AR$, there are multiple transgenic mouse models with $GABA_AR$ subunits either genetically deleted, or mutated, which has led to a better understanding of the physiological role of $GABA_AR$ isoforms (Neubauer & Kulkarni, 2006; Rudolph & Mohler, 2004). Transgenic models could facilitate a greater understanding of how a given drug may exert its effect on the $GABA_AR$ should the drug be proven to be advantageous in diabetic neuropathy.

One of the most common models of T1DM is generated by the injection of streptozotocin to destroy the β cells of the Islets of Langerhans (Agarwal, 1980;

Cefalu, 2006; Kaplan & Wagner, 2006; Ozcan *et al.,* 2008). However, streptozotocin is not always completely effective and therefore the development of consistent neuropathy cannot be guaranteed (Sullivan *et al.,* 2007). In contrast, the type-2 diabetic *ob/ob* mouse, which has an autosomal recessive nonsense mutation on chromosome 6 develops morbid obesity due to its rapacious appetite and exhibits a predictable and spontaneous neuropathic phenotype (Cefalu, 2006; Drel *et al.,* 2006; Latham *et al.,* 2009; Lindström 2009; Vareniuk *et al.,* 2007). In the *ob/ob* mouse hyper-insulinaemia develops around 3-4 weeks of age, but diabetes develops as a result of failure to increase β cell mass in response to insulin resistance induced by profound obesity (Cefalu, 2006; Lindström, 2007). It subsequently exhibits both large motor fibre and smaller sensory fibre neuropathy (Drel *et al.,* 2006; Latham *et al.,* 2009). The *ob/ob* is particularly useful for the study of neuropathic pain because it has a less severe hyperglycaemia (19mmol/l of blood) and therefore arguably provides a more clinically relevant form of diabetes than other models (Cefalu, 2006; Drel *et al.,* 2006; Latham *et al.,* 2009; Lindström, 2009; Neubauer & Kulkarni, 2006; Sullivan *et al.,* 2007). The *ob/ob* mouse develops mechanical hypersensitivity consistently by the age of 8 weeks in contrast to some of the other models, which exhibit a more severe (and less predictable) neuropathy associated with earlier sensory and motor deficits that is less clinically relevant (Drel *et al.,* 2006; Latham *et al.,* 2009; Lindström, 2009; Vareniuk *et al.,* 2007). There are conflicting reports from two different research centres stating on the one side that the *ob/ob* mouse develops thermal hypersensitivity but on the other that it develops hyposensitivity (Drel *et al.,* 2006; Latham *et al.,* 2009). This issue will be explored further in the Discussion: section 7.11.

The *ob/ob* mouse was discovered in 1949 and found to have a nonsense mutation in the gene responsible for the synthesis of leptin, thereby making the mouse totally deficient of leptin (Lindström 2007; Zhang *et al.,* 1994). Leptin is a 16kDa protein hormone that is engaged in the regulation of metabolism and appetite. Specifically, leptin is involved in the process of satiety after eating and deficiency of the hormone is associated with hyperphagia, morbid obesity and T2DM (Ahima *et al.,* 1996; Frühbeck 2006; Lindström 2009).

In contrast, the *db/db* mouse has an autosomal recessive mutation of the leptin receptor gene, which prevents leptin from activating its receptor (Chen *et al.,* 1996; Chung *et al.,* 1996). In this mouse, hyperinsulinaemia develops around 2 weeks, obesity by ~3-4 weeks and severe hyperglycaemia (>30mmol/l) around 4-8 weeks of age following β-cell failure (Bates *et al.,* 2005; Cefalu, 2006; Neubauer & Kulkarni, 2006). In common with the *ob/ob* mouse, the *db/db* mouse develops neuropathy consistently by the age of 8 weeks (Sullivan *et al.,* 2007). This condition is characterised by a significant loss of thermal sensation and profound reductions of motor and sensory nerve conduction velocity (Sullivan *et al.,* 2007). Indeed, the *db/db* mouse model appears to provide a relatively more rapid and extreme form of diabetes and neuropathy that is associated with a much-reduced lifespan (Bates *et al.,* 2005; Sullivan *et al.,* 2007).

The *ob/ob* model appears to reflect more closely the paradoxical combination of sensory thresholds observed in humans with diabetic neuropathy. The degree of neuropathy can be objectively determined using electrophysiologal studies and the microscopic examination of nerve fibres. Nevertheless, the actual levels of discomfort experienced cannot be quantified within an animal model and this is perhaps the greatest limitation of this type of experiment. Equally, it is worth noting that human experiments also struggle with this issue to an extent because of the potential difficulties inherent in the description of a subjective experience. A dimensionless visual analogue score (*i.e.* subjective severity score of pain out of ten) is frequently used but its value is limited and questionable. More recently, clinical studies of pain have started to incorporate multidimensional tools such as the McGill Pain Questionnaire, which includes emotional descriptors (Melzack, 1987).

Part 2. The $GABA_AR$

1.2.1 History of GABA & the $GABA_AR$

Gamma-aminobutyric acid (GABA) is the principle inhibitory neurotransmitter within the mammalian central nervous system (Reviewed in: Belelli & Lambert, 2005; D'Hulst *et al.,* 2009). However, the key role of GABA was not established until the second half of the twentieth century. The role of GABA in neurotransmission was investigated first in crustaceans in the 1950s and then in the mammalian nervous system in the 1960s (Bazemore *et al.,* 1956; Curtis *et al.,* 1959; Florey & McLennon, 1955; Krnjevic & Schwartz, 1967; Obata *et al.,* 1967). Technological advances such as intracellular recording microelectrodes, stimulating electrodes and multi-barrel pyrex microiontophoretic pipettes facilitated greater understanding of the significance of GABA (Curtis *et al.,* 1959). Subsequently, in the 1970s the role of GABA as a major inhibitory neurotransmitter was confirmed using bicuculline to selectively antagonise the inhibitory effects of GABA (Bennett & Balcar, 1999; Bowery *et al.,* 2004; Curtis *et al.,* 1970; Krnjevic 2004; 2010). In the same decade, intracellular recording techniques were performed on mouse spinal neurones maintained in cell culture to investigate which pharmacological agents could replicate, modulate, or antagonise, the response to GABA (MacDonald & Barker, 1977; 1978a; 1978b; See section 1.2.11 for further details about GABA pharmacology). In their landmark paper, Hamill *et al.,* (1983), used the pioneering patch-clamp technique (Neher & Sakmann, 1976) to demonstrate the presence of chloride ion channel currents that were activated by GABA and blocked by bicuculline.

Subsequent advances in cloning techniques led to the discovery that $GABA_ARs$ are comprised of distinct subunits such as α, β and γ (Pritchett *et al.,* 1989; Schofield *et al.,* 1987; See section 1.2.8 for further details on subunits). During the years that followed, the number of subunits identified by cloning grew and led to the understanding that multiple heterogenous $GABA_ARs$ existed in the mammalian nervous system (McKernan & Whiting, 1996; Olsen & Sieghart, 2008). The expression of these individual, structurally distinct $GABA_ARs$ with specialised

physiological and pharmacological functions was found to vary in different regions of the brain (Olsen & Sieghart, 2008). $GABA_A$Rs belong to the Cys loop ligand gated ion channel (LGIC) family, which includes nicotinic acetylcholine receptors, strychnine sensitive glycine receptors (see section 1.2.13) and a subtype of serotonin receptors (5-HT_3; Olsen & Sieghart, 2008).

$GABA_A$Rs are the target of many drugs used clinically including benzodiazepines (BDZs; see section 1.2.12), barbiturates and general anaesthetics, which all act to enhance receptor function (Belelli & Lambert, 2005; D'Hulst *et al*., 2009). In addition, endogenous neurosteroids such as allopregnanolone have a high affinity for $GABA_A$Rs and are involved in the maintenance of physiological neural inhibitory tone (Belelli & Lambert, 2005; D'Hulst *et al.,* 2009). Pharmacological agents that antagonise the $GABA_A$R such as bicuculline and picrotoxin are pro-convulsant (D'Hulst *et al.,* 2009; Hevers & Luddens, 1998; see section 1.2.11 for further details on the pharmacology of the $GABA_A$R).

In the early 1980s it became evident that there were two main classes of GABA receptor; those that were sensitive to bicuculline ($GABA_A$Rs) and those that were insensitive ($GABA_B$Rs; Bowery *et al.,* 1980; Hill & Bowery 1981). Despite both receptors being activated by GABA, the two receptors have entirely different structures and functional mechanisms (Bormann 2000; Ulrich & Bettler 2007). $GABA_A$Rs are LGICs, $GABA_B$Rs are G-protein coupled receptors whose structure includes seven transmembrane spanning loops (Ulrich & Bettler, 2007). $GABA_B$R activation induces G-protein subunit dissociation, which in turn can mediate a chain of events such as the inhibition of adenylate cyclase, leading to a decrease in the levels of cyclic AMP and a subsequent decrease in activation of protein kinase A (Bowery & Enna, 2000; Ulrich & Bettler, 2007). In the postsynaptic neurone, $GABA_B$Rs mediate slow inhibitory postsynaptic potentials *via* the activation of potassium ion channels, which hyperpolarise the postsynaptic membrane (Ulrich & Bettler, 2007). The $GABA_B$R agonist baclofen is a clinically useful anti-spasmodic agent, which decreases the probability that depolarisation will activate voltage-gated calcium channels, leading to a decrease in neurotransmitter release. The

hyperpolarisation thus reduces the generation of action potentials and therefore reducing muscular contraction (Bowery & Enna, 2000).

In 1984, another type of GABA receptor was discovered and given the name 'GABA$_C$R' (Drew *et al.,* 1984). 'GABA$_C$Rs' are functionally distinct from GABA$_A$Rs but due to structural similarities are now predominantly considered to be a subclass of GABA$_A$Rs known as GABA$_A$-ρRs (Alexander *et al.,* 2011; Bormann, 2000. GABA$_A$-ρRs are composed of ρ-subunits and insensitive to both bicuculline and baclofen, but they have greater affinity for GABA and can mediate strong neural inhibition. (See section 1.2.8 for further details on subunits). The GABA$_A$-ρRs are expressed much less densely than the other types of GABA$_A$ receptors and are present predominantly in the retina, cerebellum and spinal cord (Cutting *et al.,* 1991; Park *et al.,* 1999; Rozzo *et al.,* 2002).

1.2.2 GABA Synthesis

In the nervous system, glutamate is decarboxylated to GABA by glutamic acid decarboxylase (GAD; Awapara *et al.,* 1950; D'Hulst *et al.,* 2009; Madsen *et al.,* 2008; Roberts & Frankel, 1950). There are two different isoforms of GAD, with differing molecular weights: GAD 65 and GAD 67 (Conti *et al.,* 2011; Wei & Wu, 2008). The two forms of GAD have very similar molecular structures, but mediate different physiological processes. GAD 67 is widely distributed throughout the brain with its cofactor pyridoxal phosphate, while GAD 65 is localised to axonal terminals (Kaufman *et al.,* 1991). These distribution patterns suggest that the former may generate GABA to be involved in trophic or metabolic processes, while the latter may play a role in GABAergic neurotransmission (Kaufman *et al.,* 1991; Owens & Kriegstein, 2002).

1.2.3 Metabolism

The enzyme GABA α-oxoglutarate transaminase metabolises GABA to succinate semialdehyde, which is subsequently converted to succinic acid in order to enter the tricarboxylic acid cycle for oxidative metabolism (Madsen *et al.,* 2008). However, a substantial amount of GABA is repackaged into synaptic vesicles within the nerve terminal and thus recycled as a neurotransmitter (Madsen *et al.,* 2008). The transporter protein VGAT mediates the vesicular uptake of GABA by a co-transport process linked to proton exchange (McIntire *et al.,* 1997).

1.2.4 Release

GABA is released from presynaptic intracellular vesicles by the process of exocytosis. This is a rapid but highly complex process that is mediated by a superfamily of specialised proteins that are described as SNAREs (Gerst, 1999; Jahn & Scheller, 2006; Martens & McMahon, 2008). Exocytosis is initiated by calcium ion influx *via* voltage-gated calcium channels into the presynaptic nerve terminal ~1ms following an action potential. Calcium binds to synaptobrevin, which is located on the vesicular membrane and to SNAP25 and syntaxin-1, which are located on the plasma membrane (Gerst, 1999; Martens & McMahon, 2008). The binding of calcium brings the vesicle into close proximity with the plasma membrane of the presynaptic bouton, thus facilitating vesicular fusion and the neurotransmitter release (Martens & McMahon, 2008).

1.2.5 Re-uptake

Synaptic GABAergic transmission is typically rapid, but relatively short-lived. Two processes terminate these synaptic (or phasic) events: GABA diffuses away from the synaptic cleft and/or GABA transporters (GATs) mediate re-uptake. GATs differ from the other GABA transporters and consist of a 12-transmembrane spanning protein that incorporates the electrogenic process of sodium flux into its

mechanism of action. The Na^+K^+ATPase pump generates an electrochemical sodium gradient across the plasma membrane, which provides the energy for GAT to transport GABA into neurones and glia (Kanner, 2006). In mice, four different subtypes of GAT have been identified and each of these has a unique molecular structure and subsequently a different affinity for GABA (Conti *et al.,* 2011; Liu *et al.,* 1993). The GAT subtypes have varying distribution patterns within the brain and there may also be interspecies differences in subtype profiles. For instance, GAT1 is particularly localised to presynaptic nerve terminals where it is thought to contribute to the regulation of synaptic inhibition (Conti *et al.,* 2011; Durkin *et al.,* 1995). GABA transporters have an indirect but important role in neuronal inhibition and are targets for therapeutic interventions, such as anti-epileptic drugs (Madsen *et al.,* 2010).

1.2.6 Developmental role of GABA

In addition to its major role of inhibitory neurotransmitter in the adult mammalian nervous system, GABA also has a separate complex role in neurodevelopment. During development, GABA influences neuronal proliferation, migration, differentiation, synapse maturation and signalling (Ben Ari *et al.,* 2007; Di Cristo, 2007; Owens & Kriegstein, 2002). GABA is present in evolutionarily primitive organisms and may have a trophic role in that setting (Owens & Kriegstein, 2002). In early mammalian development, $GABA_AR$ activation may result in depolarisation rather than the hyperpolarisation observed in mature neurones. This situation is a consequence of a relatively high intracellular chloride concentration resulting from the delayed expression of the chloride ion transporter KCC2 (Ben Ari *et al.,* 2007; Di Cristo, 2007; Owens & Kriegstein, 2002). $GABA_AR$-mediated depolarisation in turn may be sufficient to activate voltage-gated calcium channels, which results in a rise in the concentration of calcium ions, which are integral for multiple developmental processes (Ben Ari *et al.,* 2007; Di Cristo, 2007; Owens & Kriegstein, 2002). In this way, GABA may exert apparently paradoxical but vital effects in the development of neuronal networks (Ben Ari *et al.,* 2007; Owens & Kriegstein, 2002). Indeed, in very early development GABA can mediate primary afferent

depolarisation and thus provide the earliest functional excitatory synapses prior to the establishment of glutamate synapses (Ben Ari *et al.,* 2007). In certain pathological states such as epilepsy and neuropathic pain, $GABA_AR$ activation may also result in depolarisation of spinal neurones and hippocampal neurones respectively (Coull *et al.,* 2003; Huberfeld *et al.,* 2007).

1.2.7 $GABA_A$ receptors

The $GABA_AR$ is a member of the Cys-loop superfamily of LGICs (D'Hulst *et al.,* 2009; Miller & Smart, 2010; Olsen & Sieghart, 2008). Other members of this superfamily include the serotonin ($5\text{-}HT_3$), the glycine (GlyR) and the nicotinic acetylcholine receptor (D'Hulst *et al.,* 2009; Olsen & Sieghart, 2008; Peters *et al.,* 2005). Cys-loop LGICs are pentameric structures made up of five parallel subunits that have an extracellular domain that binds the ligand and a membrane-spanning domain that forms the ion-channel (D'Hulst *et al.,* 2009; Olsen & Sieghart, 2008; Peters *et al.,* 2005). The extracellular, hydrophilic N-terminal domain contains the GABA binding site, in addition to the characteristic eponymous loop formed by a disulfide bond between two cysteine residues (Miller & Smart, 2010; Olsen & Sieghart, 2008). The membranal component comprises four α-helix transmembrane domains (M1-4), with a short extracellular C-terminus (D'Hulst *et al.,* 2009; Olsen & Sieghart, 2008; Peters *et al.,* 2005). The lining of the ion channel is formed by M2, with a potential contribution from M1 (Olsen & Sieghart, 2008). There is a relatively large loop between M3 and M4, which contributes to the ion conductance pathway and phosphorylation of this loop may be involved in the regulation of ion-channel function (Hinkle & Macdonald, 2003; Peters *et al.,* 2005).

When a ligand such as GABA binds to the interface between the extracellular domains of two adjacent subunits of the relevant Cys-loop receptor it initiates a conformational change in the receptor (Hansen *et al.,* 2005; Miller & Smart 2010). The C-loop moves inwards, trapping the agonist molecule and also induces a tilting motion of the intracellular M2 and M3 domains, which causes a widening of the

pore diameter and thus opens the ion-channel (Bocquet *et al.,* 2009; Hibbs & Gouaux, 2001; Miller & Smart, 2010).

1.2.8 Receptor subunits

Each $GABA_AR$ is composed of 5 subunits, but these subunits are drawn from a selection of 19 different types (Belelli & Lambert, 2005; Olsen & Sieghart, 2008; Simon *et al.,* 2004). Different subunit configurations permit the expression of ~20 distinct $GABA_AR$ subtypes in the mammalian CNS that exhibit unique expression patterns, have diverse physiological/pharmacological properties and mediate distinct behaviours (Belelli & Lambert, 2005; D'Hulst *et al*., 2009). In humans and rodents, the 19 identified $GABA_AR$ subunits are: α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3 (Belelli & Lambert, 2005; Möhler, 2006; Olsen & Sieghart, 2008; Simon *et al.,* 2004). The majority of native $GABA_ARs$ are composed of one γ, two α and two β subunits in a quasi-symmetrical arrangement (Belelli & Lambert, 2005; Möhler, 2006; Olsen & Sieghart, 2008; Peters *et al.,* 2005). Approximately 60% of $GABA_ARs$ *in vivo* have the configuration $\alpha1\beta2\gamma2$, while the majority of the remainder are either $\alpha2\beta3\gamma2$, or $\alpha3\beta x\gamma2$ (Belelli & Lambert, 2005; Möhler, 2006). The other subunits such as the δ, or the ε are only present in a minority of $GABA_ARs$, but they may have greater affinity for certain endogenous ligands, thus providing specialised physiological roles in certain brain regions (Belelli & Lambert, 2005; Möhler, 2006; Olsen & Sieghart, 2008).

Immunocytochemical and *in situ* hybridisation studies in rodents have revealed that individual $GABA_AR$ subunits have distinctive patterns of distribution within the CNS (Bohlhalter *et al.,* 1996; D'Hulst *et al.,* 2009; Frischy *et al.,* 1992; Pirker *et al.,* 2000; Rudolph *et al.,* 2001; Wisden *et al.,* 1992). This phenomenon enables diverse physiological and pharmacological properties of different regions of the CNS and may mediate distinct patterns of behaviour (D'Hulst *et al.,* 2009; Siegert & Sperk, 2002). The subunits α1, β1-3 and γ2 are the most widely distributed, but are still subject to regional variation. Other subunits such as α2-6, γ1 and δ appear to be confined to specific brain regions, for instance, the α2 subunit is predominantly

localised to the cerebellum and forebrain, while α3 is preferentially expressed in the nRT and dorsal horn neurones of the spinal cord and the α6 is limited to the cerebellar granule cells (Bohlhalter *et al.,* 1996; D'Hulst *et al.,* 2009; Knabl *et al.,* 2008; Pirker *et al.,* 2000; Rudolph *et al.,* 2001; Siegert & Sperk, 2002). In comparison, the cerebral cortex and ventrobasal thalamus predominantly express α1, α4, β2 and δ subunits (Pirker *et al.,* 2000; Siegert & Sperk, 2002). The distribution and function of subunits has been studied using gene 'knock-in' and 'knock-out' strategies that involve the respective substitution, or ablation, of a specific $GABA_AR$ subunit (Luscher *et al.,* 2012; Rudolph *et al.,* 2001; see section 1.3.5 for further details).

Each $GABA_AR$ subunit has a molecular mass of 40-60 kDa, which means that each complete $GABA_AR$ typically has a mass in the region of 240-290 kDa (Hevers & Luddens, 1998). Subunits are comprised of a large extracellular hydrophilic N-terminal domain that contains the GABA binding site, four hydrophobic transmembrane α-helix domains (TM1-4) and a relatively small extracellular C-terminus (Carland *et al.,* 2009; Hevers & Luddens, 1998, Olsen & Sieghert, 2008; Peters *et al.,* 2005). Chloride ions entering the external 'vestibule' of the receptor are funnelled through the narrow transmembrane pore lined by the TM2 domain, with a potential contribution from TM1 (Carland *et al.,* 2009; Olsen & Sieghert, 2008). The TM2 pore extends into an intracellular 'vestibule,' which is perforated by five narrow openings that allow the chloride ions to pass into the cytoplasm (Carland *et al.,* 2009; Olsen & Sieghert, 2008; Peters *et al.,* 2005). These openings, which constitute the only pathway for ions into the cytoplasm, are lined by the long intracellular TM3-TM4 loop (Carland *et al.,* 2009; Peters *et al.,* 2005). The TM3-TM4 loop mediates interactions with signalling factors and trafficking such as the polysynaptic clustering of $GABA_ARs$ (Luscher *et al.,* 2011; Vithlani *et al.,* 2011). Phosphorylation of key residues within the intracellular loops of β1-3 subunits and γ2 subunits may also provide a diverse and ubiquitous mechanism for the regulation of receptor activity (Jacob *et al.,* 2008; Kittler & Moss, 2003). Specifically, the TM3-TM4 loop of β subunits contains a phosphorylation site for multiple kinases, which may have an important role in ion channel regulation (Hinkle & Macdonald, 2003).

Subunit composition is of critical importance to the deactivation kinetics of GABAergic currents (Eyre *et al.,* 2012). For instance, the decay time constant τ_W of miniature inhibitory synaptic currents (mIPSCs) from $GABA_A$Rs containing the $\alpha1$ subunit and $\alpha3$ subunits is around 4-6ms and ~28ms respectively (Eyre *et al.,* 2012). Interestingly, the subunit expression observed in mature animals may be different from that of developing animals (Bosman *et al.,* 2002; Chudomel *et al.,* 2009; Fritschy *et al.,* 1994; Laurie *et al.,* 1992; Okada *et al.,* 2000). In the immature thalamus and cerebral cortex GABAergic synaptic events have longer decay times than those recorded in developed neurones (Bosman *et al.,* 2002; Okada *et al.,* 2000). These differential kinetics appear to be due to the delayed replacement of $\alpha2/\alpha3$ subunits by the $\alpha1$ subunit in these neurones (Bosman *et al.,* 2005; Fritschy *et al.,* 1994; Okada *et al.,* 2000; Peden *et al.,* 2008). However, in $\alpha1$ 'knock-out' mice (*i.e.* genetically engineered to lack the $\alpha1$ subunit) there is also a change in decay kinetics with development (Bosman *et al.,* 2005; Peden *et al.,* 2008). This developmental change will be explored further in the Results Chapter 3.

1.2.9 Phasic inhibition

$GABA_A$R-mediated synaptic transmission facilitates the rapid and precise transfer of a presynaptic signal into a postsynaptic response (Farrant & Nusser, 2005, Mody *et al.,* 1994). A presynaptic action potential triggers a local calcium influx, which facilitates the simultaneous release of thousands of GABA molecules from multiple vesicles in the presynaptic membrane. The GABA molecules bind rapidly to $GABA_A$Rs that are clustered opposite in postsynaptic groups that range in number from ten to several hundred receptors (Farrant & Nusser, 2005). This results in the near-synchronous opening of a proportion of the $GABA_A$Rs, which are usually comprised of $\alpha\beta\gamma$ subunits (Farrant & Nusser, 2005; Mody *et al.,* 1994). Under whole-cell voltage-clamp recording conditions a miniature inhibitory postsynaptic current (mIPSC) occurs when a single vesicle releases GABA molecules, which bind to postsynaptic $GABA_A$Rs almost simultaneously. However, an action potential may also trigger the release of multiple GABA-containing vesicles simultaneously,

which may activate $GABA_A$Rs at adjacent postsynaptic densities or neighbouring synapses giving rise to an IPSC (Farrant & Nusser, 2005). An important feature of synaptic inhibition is its transient nature, which is due to the rapid diffusion of GABA away from the synaptic cleft (Farrant & Nusser, 2005). The efficient gating of the postsynaptic $GABA_A$R requires two GABA molecules (Hevers & Lüddens, 1998) and this process is relatively slow in comparison to the rate of diffusion of GABA away from the area (Farrant & Nusser, 2005). Therefore, although the peak synaptic GABA concentration may be potentially saturating, not all postsynaptic $GABA_A$Rs will be occupied. This means that there is scope for other factors such as allosteric modulators of the $GABA_A$R to have a significant impact on synaptic transmission (Barberis *et al.,* 2011; Farrant & Nusser, 2005; Mody *et al.,* 1994). Although synaptic $GABA_A$Rs open near-simultaneously (resulting in a relatively rapid rising phase), they remain open for different lengths of time, which means that a typical mIPSC has an initial rapid rising phase, followed by a relatively slow 'decay' phase back to the baseline. The rate of receptor deactivation (and desensitisation) may be influenced by differences in subunit composition, or even the presence of endogenous allosteric modulators of the $GABA_A$R, which can prolong the duration of decay time and therefore prolong the duration of phasic inhibition (Barberis *et al.,* 2011; Farrant & Nusser, 2005; Mody *et al.,* 1994; Peden *et al.,* 2008).

1.2.10 Tonic inhibition

When GABA diffuses away from the synaptic cleft it may 'spillover' to activate neighbouring synapses, or even the synapses of adjacent neurones (Farrant & Nusser, 2005; Kullmann *et al.,* 2005; Mody, 2001). In addition to synaptic spillover, low ambient concentrations of GABA may exert a persistent, or 'tonic,' effect on extrasynaptic $GABA_A$Rs that is distinct from phasic activation (Farrant & Nusser, 2005; Kullmann *et al.,* 2005; Mody, 2001). Extrasynaptic $GABA_A$Rs often contain subunits such as the $\alpha 6$ or δ subunits that are less commonly observed in the synaptic setting. These subunits have greater affinity for GABA, or lower rates of desensitisation (Brickley *et al.,* 1999; Kullmann *et al.,* 2005; Mody, 2001). While

phasic activation has a crucial role in the transfer of inter-neuronal signals, tonic activation is less well understood, but appears to be important for preventing neuronal over-activation (Farrant & Nusser, 2005; Kullmann *et al.,* 2005; Mody, 2001). The presence of an endogenous extrasynaptic outward tonic current may be revealed by the application of the $GABA_A$Rs antagonist bicuculline, which under conditions of equal intra- and extra-cellular chloride concentration and a negative holding potential, produces an outward current and an associated decrease in membrane noise as the tonically active $GABA_A$Rs shut (Herd *et al.,* 2007).

1.2.11 Pharmacology

Therapeutic agents that target $GABA_A$R function are used in a variety of clinical settings including: anticonvulsants (lorazepam and phenobarbital); general anaesthetics (etomidate and propofol); anxiolytics and sedatives (diazepam and temazepam); and also to treat acute alcohol withdrawal syndrome (chlordiazepoxide; D'Hulst *et al.,* 2009; Johnston, 2005; Korpi *et al.,* 2002; Möhler, 2011). Drugs that activate the $GABA_A$R directly are termed agonists, while compounds that enhance the receptor's response to GABA, such as the benzodiazepines (BDZs) are referred to as positive allosteric modulators (D'Hulst *et al.,* 2009; Johnston, 2005). Some of these drugs such as the general anaesthetics, barbiturates and neurosteroids act as positive allosteric modulators at low concentrations to enhance the effect of GABA, but at higher concentrations activate the $GABA_A$R directly (Belelli *et al.,* 1996; Hales & Lambert, 1991; Johnston, 2005). In contrast, compounds that exert negative efficacy at the $GABA_A$R are termed allosteric inverse agonists, such as bicuculline (D'Hulst *et al.,* 2009; Johnston, 2005). Compounds that bind allosterically but are without efficacy are termed allosteric antagonists, for example, flumazenil which reverses the allosteric modulation of the $GABA_A$R by BDZs (D'Hulst *et al.,* 2009; Johnston, 2005; Möhler, 2011). Flumazenil is used clinically for the reversal of sedation associated with BDZ overdose (Veiraiah *et al.,* 2012).

The diverse array of $GABA_AR$ modulators and the distinctive effects that different compounds exert is due to the existence of multiple binding sites on the $GABA_AR$, which are influenced by subunit composition (Belelli & Lambert, 2005; Hevers & Lüddens, 1998; Siegert & Sperk, 2002). For example, the α-β interface is crucial for GABA binding, the α-γ interface is required for BDZ binding and the α-δ interface appears to mediate high affinity to GABA and certain neurosteroids (Brown *et al.,* 2002; Cromer *et al.,* 2002; D'Hulst *et al.,* 2009). The relevance of these distinct binding sites may be illustrated using examples of drugs with specific binding properties, such as etomidate, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP) and BDZs.

The anaesthetic agent etomidate has greater efficacy for $GABA_ARs$ containing the β2 or β3 subunits than β1 subunit (Belelli *et al.,* 1997). Indeed, the hypnotic effects of etomidate are inhibited in 'knock-in mice' that have point mutations in the β2, or β3, subunits, β2N265S and β3N265M respectively (Jurd *et al.,* 2003; Reynolds *et al.,* 2003). THIP is a low affinity partial agonist at receptors expressing the γ subunit (*i.e.* most $GABA_ARs$), but has high affinity for $GABA_ARs$ that express the α4β3δ subunits where it behaves as a 'superagonist' compared to GABA (Brown *et al.,* 2002; Farrant & Nusser, 2005; Krogsgaard-Larsen *et al.,* 2004). δ-$GABA_ARs$ are predominantly located at extrasynaptic locations; therefore THIP is a useful pharmacological agent for the selective activation of tonic currents (Belelli *et al.,* 2005; Farrant & Nusser, 2005).

1.2.12 Benzodiazepines

BDZs such as diazepam and chlordiazepoxide induce a conformational change in the $GABA_AR$, which favours the open state of the channel, thus increasing the efficacy of GABA, but they cannot induce chloride currents themselves (D'Hulst *et al.,* 2009; Hevers & Lüddens, 1998; Lüscher *et al.,* 2012; Macdonald & Olsen, 1994; Study & Barker, 1981). This allosteric modulation of the $GABA_AR$ mediates their sedative, anxiolytic, anticonvulsant and muscle relaxant effects (Luscher *et al.,*

2012). BDZs merit further discussion due to the important role that they have played in exploring the functional properties of the $GABA_AR$.

Classical BDZs exhibit greater activity for $GABA_ARs$ expressing the $\gamma2$ subunit, rather than the $\gamma1$, or $\gamma3$ subunits (Pritchett *et al.,* 1989) and they do not modulate $GABA_ARs$ that lack the γ-subunit, *i.e.* those that alternatively express the δ, ε, π or θ subunits (Rudolph & Knoflach, 2011). The α, but not the β, subunit is also important for BDZ binding to the $GABA_AR$ (Korpi *et al.,* 2002; Rudolph & Knoflach, 2011). For instance, $GABA_ARs$ expressing the $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ subunits are highly sensitive to diazepam, while $GABA_ARs$ expressing the $\alpha4$ or $\alpha6$ subunits are insensitive to diazepam (Rudolph & Knoflach, 2011). A single histidine residue of the $\alpha1$ subunit (H101; Wieland *et al.,* 1992) and phenylalanine and methionine residues at positions 77 and 130 respectively of the $\gamma2$ subunit (Buhr & Sigel, 1997; Sigel & Lüscher, 2011) have been identified as being key to $GABA_AR$ sensitivity to BDZs. Thus, the BDZ binding site is at the interface between $\alpha1$ and $\gamma2$ subunits, in the large extracellular domain of the $GABA_AR$ and is homologous to the binding site for GABA at the α-β interface (Lüscher *et al.,* 2012).

The histidine residue H101 within the $\alpha1$ subunit and the homologous residues $\alpha2$H101, $\alpha3$H126 and $\alpha5$H105 are essential for $GABA_AR$ modulation by diazepam (Lüscher *et al.,* 2012; Mohler, 2011). Behavioural and pharmacological investigations of 'knock-in' mice, where an arginine residue replaced the relevant histidine residue, enabled the study of the properties of individual α subunits (Luscher *et al.,* 2012; Mohler, 2011). The $\alpha1$ subunit mediates the sedative and amnesic effects of diazepam, while the $\alpha2$ and $\alpha3$ subunits mediate the anxiolytic effect of the drug (Luscher *et al.,* 2012). In addition, the $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subunits mediate the myorelaxant effects of diazepam (Luscher *et al.,* 2012). $\alpha2$ and $\alpha3$ 'knock-in' mice have been used to demonstrate that the $\alpha2$ and $\alpha3$ subunits of $GABA_ARs$ mediate the analgesic effect of BDZs (Knabl *et al.,* 2008). Indeed, the '$\alpha1$-sparing' BDZ-site ligand L-838,417 was highly effective against both inflammatory and neuropathic pain, without the usual BDZ side effects of sedation, ataxia and

tolerance by targeting neurones expressing the α2 and α3 subunits, but was ineffective in the 'knock-in' mice (Knabl *et al.,* 2008).

1.2.13 The Glycine receptor

As mentioned above, the glycine receptor (GlyR) is also a member of the Cys-loop superfamily of ligand-gated ion-channels (LGICs; Connolly & Wafford, 2004; Lynch, 2009; Miller & Smart, 2010). The GlyR is an important mediator of neural inhibition within the spinal cord, brainstem and retina, but the receptor is also expressed at relatively low levels in other regions (Baer *et al.,* 2009; Lynch, 2009). Functional GlyRs are comprised of five membrane-spanning subunits (α1-α4 and β) that form a central chloride ion-channel (Baer *et al.,* 2009; Lynch, 2009). The receptor may be constructed solely from a single isoform of α subunits (homomeric), as is the case early in development, or it may contain α and β subunits (heteromeric; Baer *et al.,* 2009; Lynch, 2009). GlyRs may be distinguished from $GABA_A$Rs pharmacologically, because the former is sensitive to strychnine, while the latter is sensitive to bicuculline (Callister & Graham, 2010; Inquimbert *et al.,* 2007; Keller *et al.,* 2001; Mitchell *et al.,* 2007). Indeed, these agents may be used to distinguish between the two receptors in electrophysiological studies of the dorsal horn neurones of the pain pathway (Inquimbert *et al.,* 2007; Keller *et al.,* 2001; Mitchell *et al.,* 2007). In parallel with the $GABA_A$R, the GlyR plays an important inhibitory role in the dorsal horn of the spinal cord and potentially may be modulated by specific neuroactive steroids such as minaxolone (Mitchell *et al.,* 2007). Indeed, the GlyR may also prove to be a therapeutic target for diseases associated with the lack of neural inhibition such as neuropathic pain syndromes (Inquimbert *et al.,* 2007; Keller *et al.,* 2001; Lynch, 2009).

Part 3. Neurosteroids

1.3.1 Overview

Steroid hormones are synthesised in peripheral endocrine glands such as the ovaries and the adrenal glands and regulate protein transcription at the level of the cellular nucleus (Reviewed in: Belelli & Lambert, 2005; Paul & Purdy, 1992; Picard & Yamamoto, 1987). They exert important long-term reproductive and neuroendocrine effects that may take hours, or days, to manifest (McEwen, 1991; Paul & Purdy, 1992; Sierra, 2004). However, metabolites of the steroid hormone progesterone have been shown to have rapid anaesthetic effects that are inconsistent with a genomic mechanism of action (Belelli & Lambert, 2005; McEwen, 1991; Paul & Purdy, 1992; Selye, 1941). Alphaxalone, a synthetic steroid structurally related to these progesterone metabolites was subsequently found to be an allosteric modulator of the $GABA_AR$ (Barker *et al.,* 1987; Belelli & Lambert, 2005; Cottrell *et al.,* 1987; Harrison & Simmonds, 1984). At low concentrations the progesterone-related neuroactive steroids typically exert their effects by increasing the sensitivity of the $GABA_AR$ to GABA, but at high concentrations may activate the $GABA_AR$ directly (Callachan *et al.,* 1987; Peters *et al.,* 1988; Shu *et al.,* 2004).

Traditionally it was believed that neuroactive steroids were synthesised only in peripheral endocrine glands in response to physiological stimuli such as pregnancy, or emotional stress, and subsequently crossed the blood-brain barrier (Baulieu *et al.,* 2001; Paul & Purdy, 1992; Purdy *et al.,* 1991). However, it was discovered that neurones and glial cells within the CNS (including the cortex and thalamus) were able to synthesise neuroactive steroids, which were able to influence neuronal function in a paracrine, or autocrine, manner (Agis-Balboa *et al.,* 2006; Baulieu *et al.,* 2001; Do Rego *et al.,* 2009; Lambert *et al.,* 2003). Neurosteroids play a crucial role in the development and function of the CNS and they are implicated in the proliferation, differentiation, activity and survival of neurones (Belelli & Lambert, 2005; Do Rego *et al.,* 2009; Mellon, 2007; Mellon *et*

al., 2001). In addition, changes in neurosteroid levels occur with certain forms of depression, epilepsy, schizophrenia, pregnancy, acute stress and Alzheimer's disease (Belelli *et al.,* 2006; Gunn *et al.,* 2011; Luchetti *et al.,* 2011; Reddy, 2010).

1.3.2 Neurosteroid binding site

Radioligand binding and functional studies have demonstrated that neurosteroids enhance $GABA_AR$ function by binding to a site(s) that is distinct from the BDZ and barbiturate binding sites (Callachan *et al.,* 1987; Gee *et al.,* 1988; Peters *et al.,* 1988). Hosie *et al.,* (2006) used molecular biology techniques in insects to indentify two discrete putative binding sites for neurosteroids in the transmembrane domain of the $GABA_AR$ that mediate their potentiating effects and direct activation. Specifically, neurosteroids potentiate GABA from a cavity within the α subunit domain, while direct activation is *via* the α-β interface (Hosie *et al.,* 2006). Using tight-seal cell attached electrophysiological recordings in which direct drug access to the membrane was prevented Akk *et al.,* (2005) demonstrated that steroids can reach the $GABA_AR$ either through plasma membrane lateral diffusion or *via* intracellular routes. In addition, a membrane-impermeable neuroactive steroid was only able to activate the $GABA_AR$ when applied intracellularly (Akk *et al.,* 2005). However, only the bath application of a membrane permeable neurosteroid potentiated the $GABA_AR$, suggesting that the neurosteroid either partitioned into the membrane, or diffused into the cell (Akk *et al.,* 2005). The demonstration that intracellular neurosteroid is effective in modulating the $GABA_AR$ is consistent with the hypothesis that neurosteroids may act in an autocrine fashion (Akk *et al.,* 2009; Chisari *et al.,* 2009).

1.3.3 Synthesis and metabolism

The initial rate-limiting step in the synthesis of neurosteroids is the translocation of cholesterol across the mitochondrial membrane by the 18 kDa translocator protein (TSPO; Rupprecht *et al.,* 2010; Sierra, 2004; see Figure 2). TSPO functions

in a coordinated manner with the steroidogenic acute regulatory (StAR) protein to shuttle the hydrophobic cholesterol molecule across the aqueous intermembrane space of the mitochondrial membrane (Do Rego *et al.,* 2009; Sierra, 2004). Cholesterol is then converted to pregnenolone by the cholesterol side-chain cleavage enzyme P450scc, which is located in the inner mitochondrial membrane (Do Rego *et al.,* 2009; Mellon *et al.,* 2001; Rupprecht *et al.,* 2010; Sierra, 2004). Pregnenolone is the precursor for numerous synthesis pathways that result in the production of a wide range of neurosteroids, each with differing functions, and including: oestradiol, testosterone and progesterone (Baulieu *et al.,* 2001; Do Rego *et al.,* 2009; Mellon *et al.,* 2001). Pregnenolone exits the mitochondria and undergoes a stepwise series of specific enzymatic conversions to produce allopregnanolone. The pathway is as follows: Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD); Progesterone is converted to 5α-dihydroxyprogesterone (DHP) by 5α-reductase (5α-R); DHP is converted to allopregnanolone (5α3α) by 3α-hydroxysteroid dehydrogenase (3α-HSD).

In addition, the active compound allopregnanolone may be converted back to DHP, or to the inactive epiallopregnanolone (3β5α) (Belelli & Lambert, 2005; Mellon *et al.,* 2001; Rupprecht *et al.,* 2010; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003; see Figure 3). The neurosteroid synthesis pathways are complex, not fully understood and the expression of different subtypes of the relevant enzymes appears to be species-specific. For example, two types of 5α-R and four types of 3α-HSD have been identified and each of these isoforms has a different capacity to modulate the levels of active steroid hormones (Agis-Balboa *et al.,* 2006, Li *et al.,* 1997; Pelletier *et al.,* 2004; Penning *et al.,* 2000; Stoffel-Wagner, 2003; Tsuruo, 2005).

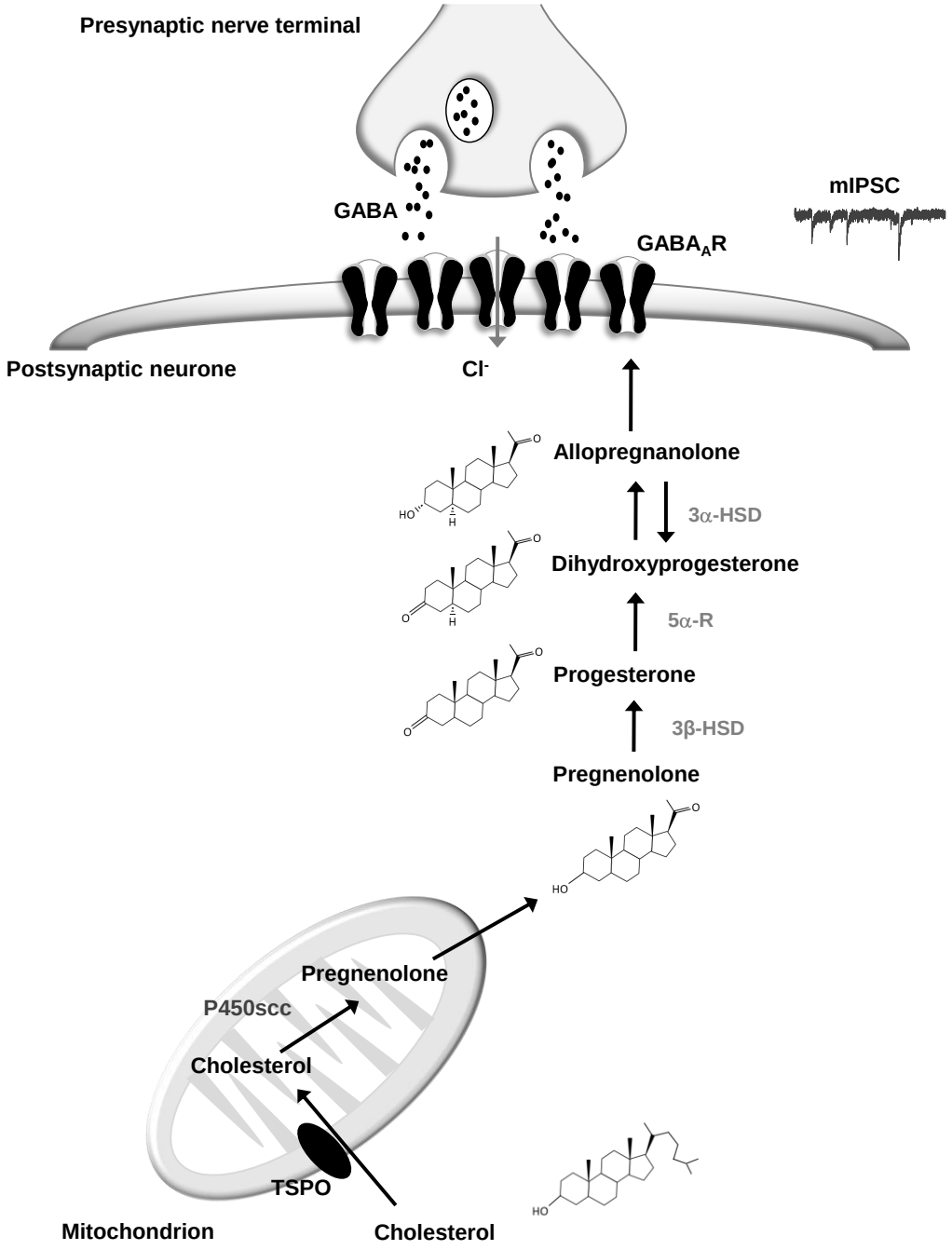

Figure 2 Modulation of the GABA$_A$R by endogenous neurosteroids.

Cholesterol is taken through the mitochondrial membrane by the translocator protein (TSPO) where it is converted to pregnenolone by the cytochrome P450 side chain cleavage enzyme. Pregnenolone undergoes stepwise enzymatic conversion to other neurosteroid compounds and ultimately allopregnanolone which modulates GABA$_A$R function. Neurosteroids may act *via* paracrine, or autocrine, mechanisms. Postsynaptic GABA$_A$Rs are activated by GABA that has been released from vesicles in the presynaptic nerve terminal. GABA induces a conformational change in the GABA$_A$R, which 'opens' its central pore, allowing the passage of chloride ions and the generation of mIPSCs. The negatively charged chloride ions typically induce hyperpolarisation of the neuronal membrane, which is associated with neuronal inhibition. Neurosteroids such as allopregnanolone enhance GABA$_A$R function and therefore facilitate neural inhibition.

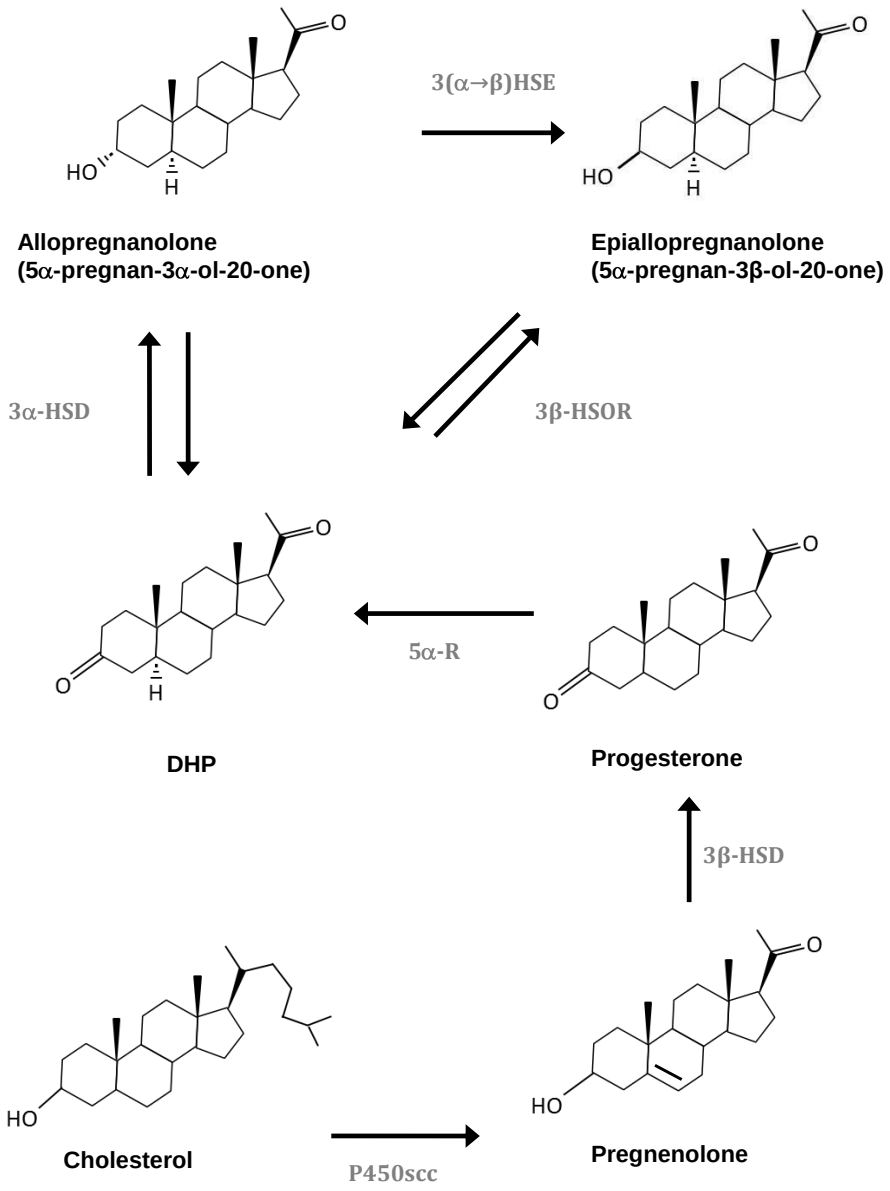

Figure 3 Neurosteroid synthesis pathway

Allopregnanolone is synthesised from neurosteroid precursors by a sequence of enzymatic conversions. These neurosteroids have a characteristic steroidal structure composed of three 6-member and one 5-member carbon rings. Hydroxyl- and methyl- groups attached to specific locations are responsible for selective activity at receptors. Cholesterol is cleaved by the enzyme cytochrome P450scc within the mitochondria to form pregnenolone, which diffuses into the cytoplasm. Cytoplasmic pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD), which is in turn reduced to DHP by 5α-reductase (5α-R). DHP is converted to allopregnanolone by 3α-HSD, which also catalyses the back-conversion reaction. The $GABA_AR$-modulatory compound allopregnanolone may be metabolised by 3(α→β) hydroxysteroid epimerase to the inactive compound epiallopregnanolone (Adapted from Schumacher *et al.,* 2012).

The heterogeneous nature of enzyme expression can lead to regional (and species) variation in the levels of a given neurosteroid and may therefore affect its availability to modulate the $GABA_AR$. For this reason, neurosteroid mediated GABAergic inhibitory tone may be relatively high in one region of the CNS, but less so in another (Belelli & Lambert, 2005; see section 1.3.4 for further details). In addition, pharmacological agents may be used to target different stages of the neurosteroid synthesis pathway to facilitate the study of neurosteroids and their precursors in the physiological setting as well as in pathological states and in neurodevelopment. The perturbation of neurosteroid levels is associated with numerous neuropathologies, therefore manipulation of their synthesis and metabolism could potentially be of therapeutic benefit (Gunn *et al.,* 2011; Luchetti *et al.,* 2011; Reddy, 2010; Rupprecht *et al.,* 2010).

Numerous synthetic ligands exist for mitochondrial TSPO, including emapunil (XBD173), etifoxine, diazepam and 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline carboxamide (PK-11195; Giatti *et al.,* 2009; Papadopoulos & Lecanu, 2009; Rupprecht *et al.,* 2010). The TSPO is considered to be a promising therapeutic target for the treatment of nerve injury and anxiety disorders *via* the enhancement of neurosteroidogenesis (Girard *et al.,* 2008; Rupprecht *et al.,* 2009, 2010). However, a TSPO ligand still requires the presence of all the key enzymes downstream in order for active neurosteroids such as allopregnanolone to be produced. The function of these enzymes has been the subject of numerous investigations: Finasteride inhibits the enzyme 5α-reductase (5α-R), which converts progesterone to DHP (which is subsequently converted to allopregnanolone; Aggarwal *et al.,* 2010). Finasteride does not have a direct effect on synaptic $GABA_ARs$, but pre-treatment of the brain slice with finasteride can prevent the conversion of progesterone into the GABA-active neurosteroid metabolites in the hippocampal CA1 neurones of mature mice (Sanna *et al.,* 2004). Interestingly, finasteride spinal slice treatment has been shown to reduce the duration of synaptic GABAergic events in the spinal LII neurones of immature rats, but had no effect on mature rats (Inquimbert *et al.,* 2007; Keller *et al.,* 2004). A similar phenomenon was observed in the cortical neurones of immature mice that were treated with another 5α-R inhibitor, 17β-17-[bis (1methylethyl) amino

carbonyl] androstane-3,5-diene-3-carboxylic acid (SKF 105111; Puia *et al.,* 2003). Interestingly, when SKF 105111 was given by an *in vivo* intraperitoneal injection it reduced the endogenous concentration of allopregnanolone to a greater extent than when the brain slice tissue was incubated with the drug; 80 percent and 30 percent reductions respectively (Puia *et al.,* 2003). These findings are consistent with the hypothesis that there is an endogenous neurosteroid tone in immature animals that has a significant modulatory effect on the $GABA_AR$ (Keller *et al.,* 2004).

Medroxyprogesterone acetate (provera) and indometacin have been used as inhibitors of 3α-HSD, the enzyme that converts DHP into the active compound allopregnanolone (Askonas *et al.,* 1991; Belelli & Herd, 2003; Belelli & Lambert, 2005; Duax *et al.,* 1978; Hori *et al.,* 2006; Sunde *et al.,* 1982). 3α-HSD also inhibits the back reaction of allopregnanolone to DHP, thus inhibition of this enzyme has been shown to enhance the effect of exogenously applied allopregnanolone in dentate gyrus neurones of rats (Belelli & Herd, 2003). Interestingly, in the same dentate gyrus neurones, the synthetic neurosteroid ganaxolone had a greater effect on GABAergic synaptic events than allopregnanolone (Belelli & Herd, 2003). Ganaxolone is a metabolically stable synthetic analogue of allopregnanolone, the molecular structure of which differs only by having an extra methyl group *i.e.* 3β (adjacent to the hydroxyl group) that prevents oxidation to an inactive form (Carter *et al.,* 1997; see Figure 3).

The cyclodextrins (CDs) have been used as pharmacological tools to study neurosteroids. The CDs are relatively large cyclic oligomers of glucose, which are barrel-shaped molecules with a hydrophilic exterior and a hollow lipophylic interior (Davis & Brewster, 2004; Adam *et al.,* 2002; see Figure 4). These characteristics mean that CDs are water soluble, but are able to sequester smaller lipophilic molecules including certain neurosteroids (Shu *et al.,* 2004, 2007). As a result of being trapped inside the CD molecule the neurosteroid agent is unable to exert an effect at the plasma membrane, such as the modulation of the $GABA_AR$. CDs may be characterised by the size of the hydrophobic pore, which is determined by the number of glucose residues it contains.

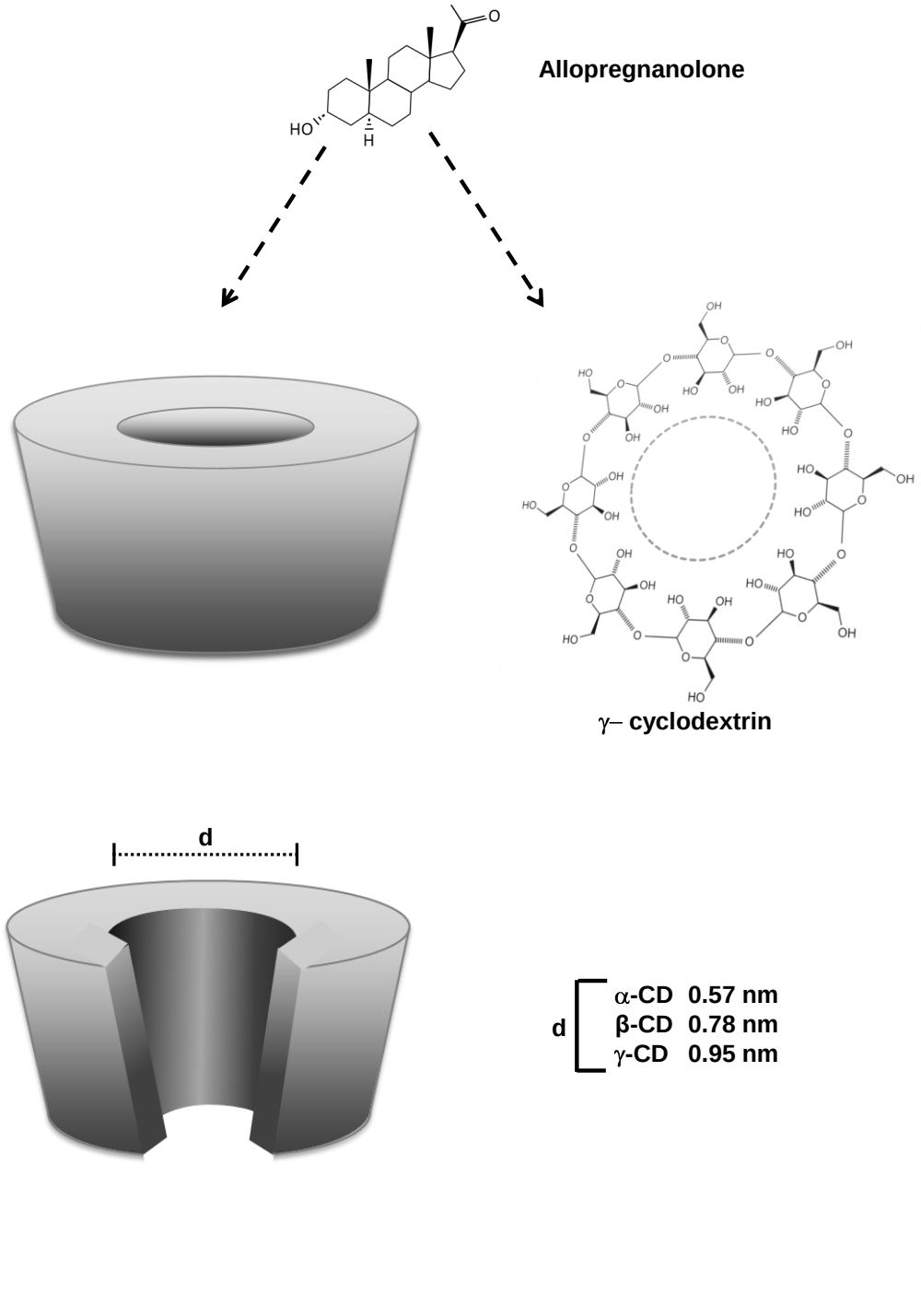

Figure 4 Cyclodextrins sequester neurosteroids.

The cyclodextrins (CDs) are relatively large cyclic oligomers of glucose, barrel-shaped molecules with a hydrophilic exterior and a hollow lipophilic interior. CDs are water soluble, but are able to sequester smaller lipophilic molecules including certain neurosteroids such as allopregnanolone. As a result of being trapped inside the CD molecule the neurosteroid compound is unable to exert an effect at the plasma membrane, such as the modulation of the $GABA_AR$. The three principal types of CD that have been described in the literature are the α-CD hexamer, the β-CD heptamer and the γ-CD octomer, these are characterised by the size of their hydrophobic pore, which is determined by the number of glucose residues present.

The three principal types of CD that have been described in the literature are the α-CD hexamer, the β-CD heptamer and the γ-CD octomer (Cooper *et al.,* 2005; Davis & Brewster, 2004). However, there are numerous possible CD configurations based on variation in ring-size and composition. γ-CD does not appear to modulate the $GABA_AR$ directly (Shu *et al.,* 2004, 2007). Paradoxically, in addition to removing endogenous lipophilic compounds from their potential target, the CDs may be used as complexing agents to solubilise lipophilic agents in aqueous solution (Brewster & Loftsson, 2007). Neuroactive steroids may be sequestered inside the lipophilic interior of CDs, thus allowing for injection *in vivo* (Davis & Brewster, 2004). Once injected, the neurosteroids are able to leave the CD molecule due to the favourable concentration gradient, or be displaced by an endogenous lipid (Brewster & Loftsson, 2007). This property is useful because oil-based vehicles may cause localised inflammation and irritation at the injection site. CDs have various current applications, including the sequestration of the muscle relaxant agent rocuronium from the circulation in order to reverse muscle relaxation rapidly (Adam *et al.,* 2002; Cooper *et al.,* 2005).

1.3.4 Localisation and distribution

Immunohistochemical studies have investigated the cellular distribution patterns of the enzymes involved in the neurosteroid synthesis pathway, however, a somewhat inconsistent picture has emerged. Melcangi *et al.,* (1990) found that 5α-R activity was preserved in neurones, oligodendrocytes and astrocytes isolated from rat brains by density ultracentrifugation and also in neurones and glial cells grown in cultures. Subsequent immunohistochemical studies on the rat brain revealed 5α-R to be expressed in glial cells, while studies on the mouse brain found that 5α-R only appeared to be present in neurones (Agis-Balboa *et al.,* 2006; Pelletier *et al.,* 1994; Tsuruo, 2005). The reason for this discrepancy is uncertain but may be due to species-specific isoforms. A study using antibody-immunolabelling of allopregnanolone in rats found the neurosteroid had a wide distribution pattern, but that it only appeared to be present in neurones and not glial cells (Saalmann *et al.,* 2007). Mass spectrometry and liquid, or gas

chromatography, have identified several free neuroactive steroids in the rat brain including allopregnanolone, along with their respective precursors (Caruso *et al.,* 2008; Ebner *et al.,* 2006). Separately, the GABAergic Purkinje cells of the cerebellum have been shown to synthesise progesterone *de novo* from cholesterol and to convert progesterone to allopregnanolone (Tsutsui, 2008). The neurosteroid-binding site appears to be within the transmembrane component of the $GABA_AR$ and therefore accessible from within the cell or *via* external lateral diffusion (Akk *et al.,* 2005). The high lipophilicity of neurosteroids is consistent with a proposed autocrine mechanism of $GABA_AR$ activation (Akk *et al.,* 2009; Chisari *et al.,* 2009).

1.3.5 Modulation of the $GABA_AR$

Neurosteroids may modulate the $GABA_AR$ at presynaptic, postsynaptic and extrasynaptic locations (Kullmann *et al.,* 2005). The principle effect of neurosteroids on synaptic $GABA_ARs$ is to increase the duration of inhibitory postsynaptic currents (IPSCs) by prolonging the channel opening time (Herd *et al.,* 2007; Mitchell *et al.,* 2008; Zhu & Vicini, 1997). Interestingly, the magnitude of neurosteroid effect on synaptic $GABA_ARs$ is neurone specific (Cooper *et al.,* 1999; Harney *et al.,* 2003; Koksma *et al.,* 2003; Vicini *et al.,* 2002). Certain cerebellar and hippocampal neurones are sensitive to nanomolar concentrations of neurosteroids such as allopregnanolone, while hypothalamic magnocellular neurones may require micromolar concentrations of such steroids for modulation of $GABA_ARs$ (Cooper *et al.,* 1999; Harney *et al.,* 2003; Koksma *et al.,* 2003; Vicini *et al.,* 2002). These differences in sensitivity may be related to the interaction of numerous factors including subunit composition, phosphorylation and metabolism (Belelli & Lambert, 2005).

Subunit composition appears to have a modest but potentially physiologically significant effect on the sensitivity of the $GABA_AR$ to neurosteroids. The function of recombinant $GABA_ARs$ containing the $\alpha1$ or the $\alpha3$ subunit were enhanced by concentrations of allopregnanolone that were more than ten times less than those

required for receptors containing the α2 or α4-6 subunits (Belelli *et al.,* 2002). $GABA_A$Rs containing the δ subunit, rather than the γ2 subunit, exhibit the greatest magnitude of response to neurosteroids (Belelli *et al.,* 2002; Brown *et al.,* 2002; Mihalek *et al.,* 1999; Wohlfarth *et al.,* 2002). This may be important in the mediation of tonic inhibition where $GABA_A$Rs are exposed to low ambient concentrations of GABA (Belelli & Lambert, 2005; Farrant & Nusser, 2005). Indeed, δ 'knock-out' mice have a reduced behavioural response to the anxiolytic, hypnotic and convulsant effects of neurosteroids (Mihalek *et al.,* 1999).

In keeping with this behavioural study, electrophysiological investigations have demonstrated that the neurosteroids allopregnanolone and tetrahydrodeoxycorticosterone (5α-THDOC) have greater efficacy at $GABA_A$Rs expressing the δ subunit (Belelli *et al.,* 2002; Brown *et al.,* 2002; Glykys *et al.,* 2007; Wohlfarth *et al.,* 2002). In addition, the relatively low efficacy of GABA interacting at δ $GABA_A$Rs could be enhanced to high efficacy by neurosteroids modulating these receptors (Bianchi & Macdonald, 2003). Whole-cell voltage-clamp studies on cerebellar and dentate gyrus granule cells within brain slices found that 5α-THDOC enhanced tonic, but not phasic inhibitory currents (Stell *et al.,* 2003). In the same study, the magnitude of the tonic current in both dentate gyrus and cerebellar granule cells of δ 'knock-out' mice was reduced *c.f.* WT. Furthermore, the residual tonic current was not enhanced by 5α-THDOC (Stell *et al.,* 2003). These findings indicate that interaction of neurosteroids and δ $GABA_A$Rs is complex and other factors such as phosphorylation and local metabolism may be influential in the neurosteroid modulation of the $GABA_A$R (Belelli & Lambert, 2005; Brussaard *et al.,* 2000; Harney *et al.,* 2003; Hinkle & Macdonald, 2003; Koksma *et al.,* 2003).

Presynaptic $GABA_A$R activation can increase, or decrease, the frequency of postsynaptic events depending on the nature of the chloride ion gradient across the membrane (Kullmann *et al.,* 2005). If the intracellular chloride concentration is relatively high, $GABA_A$R activation could induce depolarisation of the presynaptic membrane. This change in the membrane potential could be sufficient to activate voltage-gated calcium channels and lead to an increased release of

neurotransmitter (Haage *et al.,* 2002, 2005; Herd *et al.,* 2007; Kullmann *et al.,* 2005).

Phosphorylation of the $GABA_AR$ can alter its sensitivity to neurosteroids in a complex and variable manner that is yet to be elucidated fully (Brussaard *et al.,* 2000; Harney *et al.,* 2003; Hinkle & Macdonald, 2003; Hodge *et al.,* 1999; Koksma *et al.,* 2003). A specific example of the transient effect that phosphorylation may have on the $GABA_AR$ has been observed in rats in association with parturition. In pregnant rats, $GABA_ARs$ mIPSCs of oxytocin-secreting magnocellular neurones of the hypothalamic supraoptic nucleus are sensitive to neurosteroids (Brussaard *et al.,* 2000; Koksma *et al.,* 2003). However, on the day of parturition, the endogenous neurosteroid tone decreases significantly and the $GABA_ARs$ become steroid-insensitive. The net result of this process is that a physiological inhibitory brake is removed, thus allowing the release of oxytocin (Brussaard *et al.,* 2000; Koksma *et al.,* 2003). This change to inhibition is of physiological relevance because oxytocin plays a crucial role in parturition. The oxytocin surge is essential for the optimal timing of parturition, myometrial contractility and also lactation (Blanks & Thornton, 2003). The change in synaptic $GABA_AR$ sensitivity to neurosteroids may be mimicked by the manipulation of protein kinase C (PKC) and phosphatases (Koksma *et al.,* 2003).

1.3.6 Neurodevelopment

During early post-natal development a significant reduction in the duration of the decay time of synaptic $GABA_AR$ events has been described in numerous regions of the CNS including: the spinal cord, thalamus, cerebellum, dentate gyrus, hypothalamus and cerebral cortex (Brickley *et al.,* 1996; Draguhn & Heinemann, 1996; Dunning *et al.,* 1999; Keller *et al.,* 2004; Peden *et al.,* 2008; Schlichter *et al.,* 2006; Tia *et al.,* 1996). The physiological impact of this process is thought to enhance the precision of motor coordination, sensory perception and cognitive

functioning (Takahashi, 2005). Changes in subunit composition with maturation, such as incorporation of the α1 subunit result in significantly faster decay kinetics of GABAergic synaptic events (Fritchy *et al.,* 1994; Laurie *et al.,* 1992; Okada *et al.,* 2000; Vicini *et al.,* 2001). However, subunit change is not the only factor to influence the exponential decay time of $GABA_AR$-mediated mIPSCs. Indeed, dorsal horn neurones of the spinal cord exhibit shorter mIPSC decay times with development, P6-8, P13-23 and >P30 without the incorporation of the α1 subunit (Keller *et al.,* 2004). In addition, it has been observed that neurones from mice that have been genetically modified to lack the gene responsible for the α1 subunit of the $GABA_AR$ still exhibit shorter mIPSC decay times with development (Bosman *et al.,* 2002; Peden *et al.,* 2008).

The reduction of $GABA_AR$ mIPSC decay times with development is paralleled by a reduction in the level of endogenous neurosteroids within the CNS (Keller *et al.,* 2004; Grobin & Morrow, 2001; Mellon, 2001). The relative decline of neurosteroid levels is likely due to changes in the expression of 3α-HSD and 5α-R that have been observed within the CNS (Griffin *et al.,* 2004; Kellogg & Frye, 1999; Mellon, 2001). The exact physiological significance of these changes is uncertain, but neurosteroids have numerous functions within the developing nervous system including proliferation, differentiation, activity and survival of nerve cells (Belelli & Lambert, 2005; Do Rego *et al.,* 2009; Mellon, 2007; Mellon *et al.,* 2001). The precise mechanisms by which allopregnanolone mediates neurodevelopmental actions is uncertain, but $GABA_AR$-mediated depolarising currents (due to the delayed expression of the chloride ion transporter KCC2) and a consequent increase of intracellular calcium levels by activating voltage-gated calcium channels is a potential mechanism (Blaesse *et al.,* 2009; Ben-Ari *et al.,* 2007). Therefore, the modulation of $GABA_ARs$ by allopregnanolone may result in the activation of signalling pathways mediated by calcium (Keller *et al.,* 2004; Wang & Brinton, 2008).

In order to understand the role of neurosteroids in development it may be useful to consider a disease associated with a neurosteroid deficit. The neurodegenerative condition known as Niemann-Pick disease type C is

characterised by the accumulation of neuronal cholesterol, severe progressive CNS dysfunction and death in childhood (Griffin *et al.,* 2004). In a mouse model of Niemann-Pick disease type C the ability to synthesise allopregnanolone across the developmental spectrum is reduced and the neonatal administration of allopregnanolone delayed the onset of neurological symptoms (Griffin *et al.,* 2004).

1.3.7 Neurosteroids, pain and the $GABA_AR$

A loss of physiological inhibitory tone is associated with hypersensitivity symptoms such as allodynia and hyperalgesia (Chen & Pan, 2002; Munro *et al*., 2009; Zeilhofer, 2008). The $GABA_AR$ is the major inhibitory receptor in the mammalian nervous system and as such it mediates inhibitory tone throughout the pain pathway (Munro *et al*., 2009, 2011; Zeilhofer, 2008). Reduced GABAergic inhibition within the dorsal horn of the spinal cord may be implicated in the development of hypersensitivity (Knabl *et al*., 2008; Munro *et al*., 2009; von Hehn *et al*., 2012; Zeilhofer, 2008). Therefore, pharmacological agents that enhance $GABA_AR$ function could be useful to counteract lost inhibitory tone (Knabl *et al*., 2008; Munro *et al*., 2009; Zeilhofer *et al*., 2009). Neurosteroids such as allopregnanolone are potent allosteric modulators of this receptor (Belelli & Lambert, 2005; Callachan *et al.,* 1987). Indeed, an upregulation in the production of endogenous neurosteroids within the spinal cord in response to peripheral inflammation has been shown to have an analgesic effect (Poisbeau *et al*., 2005, Schlichter *et al*., 2006). The analgesic effect could be suppressed by the administration of finasteride to inhibit the enzyme 5α-R, which converts progesterone to its more active metabolites (Poisbeau *et al*., 2005).

Neurosteroids such as progesterone have been studied for their potentially protective effects for a number of different neuropathologies including stroke, brain and spinal injuries (Mensah-Nyagan *et al.,* 2009; Stein, 2008). Although there have been some promising preclinical studies, the mechanism of action is not fully understood and they have yet to be translated into useful clinical therapies (Leonelli *et al.,* 2007; Mensah-Nyagan *et al.,* 2009; Roglio *et al.,* 2008; Stein, 2008).

In rats given chemotherapy, chronic administration of the neurosteroids progesterone, or allopregnanolone, were able to prevent the development of painful peripheral neuropathy (Meyer *et al.,* 2010, 2011b).

The effects of progesterone-derived neurosteroids and their interaction with the $GABA_AR$ has not yet been described in the setting of type-2 diabetes mellitus, but ostensibly would seem to be a logical area to study. Diabetic neuropathy has a large impact on society with limited management options. Mouse models that replicate the obesity, hyperglycaemia and neuropathy observed in humans have now been characterised (Drel *et al.,* 2006; Latham *et al.,* 2009; Lindstrom *et al.,* 2007; Sullivan *et al.,* 2007). Liquid chromatography and tandem mass spectrometry studies have demonstrated that the neurosteroid precursors pregnenolone and progesterone are decreased in the cerebral cortex, cerebellum and spinal cord of mice with streptozotocin-induced diabetes mellitus (Caruso *et al.,* 2008).

The potential role of neurosteroids in painful diabetic neuropathy may be approached using a number of techniques, including: 1) Electrophysiological study of pain pathway neurones to characterise changes in $GABA_AR$ function and neurosteroid tone at different stages of development and in animals with diabetic neuropathy. 2) Behavioural characterisation of mechanical and thermal nociceptive thresholds in WT and diabetic mice before and after neurosteroid administration.

Hypotheses

1. Synaptic $GABA_AR$ mIPSCs change with development in pain pathway neurones.
2. The developmental change to $GABA_AR$ mIPSCs is related, at least in part, to fluctuations in the levels of endogenous neurosteroid.
3. The neurosteroid tone may be altered in pathological states such as in mice with diabetic neuropathy.
4. Neuropathic sensitisation may be due to a reduction of physiological GABAergic inhibitory tone.
5. Replacement of endogenous neurosteroids by synthetic neuroactive steroids may be analgesic.

Chapter 2. Methods

2.1 Electrophysiology

In the twentieth century, the field of electrophysiology evolved in parallel with advances in electric circuitry. A major breakthrough came in 1976 when Neher and Sakmann developed the patch-clamp technique, for which they would later be awarded the Nobel Prize for Physiology (Hamill *et al.,* 1981; Neher & Sakmann, 1976). The whole-cell patch-clamp method involves placing the hollow tip of a glass microelectrode directly onto the surface of a neurone under the microscope in order to make real-time electrical recordings. Gentle suction facilitates the formation of a tight (giga-Ohm) seal between the glass electrode and the cell membrane. This versatile and sensitive technique is technically challenging to perform, but can yield important insights into ion-channel function. An electrical circuit is set up that incorporates the neurone, microelectrode, current monitoring system and a specialised amplifier (Figure 5). Within the circuit, the resistance is fairly constant and the voltage may be 'clamped' at a specific point, thus changes to the current associated with ionic flux may be studied using Ohm's law ($I = V/R$). When an agonist binds to a synaptic receptor it induces a conformational change, which opens the channel allowing ions to travel across the membrane according to their electrochemical gradient. This process may be manipulated pharmacologically and the effects observed using electrophysiological techniques.

This study focuses on the mIPSCs of $GABA_A$Rs that are induced only by the mono-vesicular release of GABA at single synapses. This is because action potential driven multi-vesicular GABA release is often not simultaneous, therefore the resultant IPSCs are summated and difficult to interpret accurately. In order to isolate the postsynaptic $GABA_A$R mIPSCs it is necessary to use kynurenic acid and strychnine to block glutamate and glycine receptors respectively and tetrodotoxin is used to prevent voltage activated sodium channel-mediated action potentials and hence prevent evoked IPSCs.

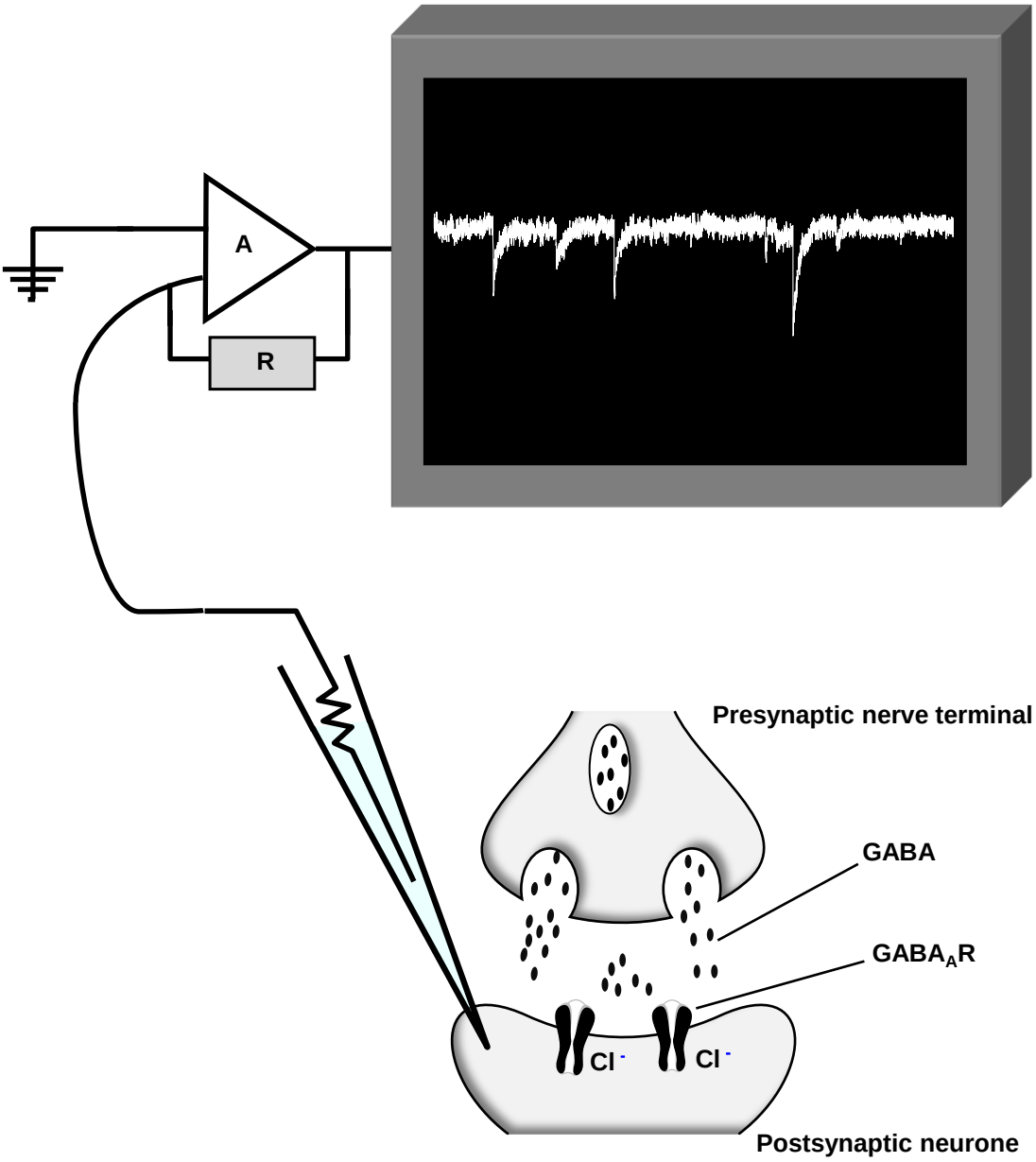

Figure 5 Simplified schematic illustrating the approach to obtaining electrophysiological recordings from pain pathway neurones.

A glass microelectrode with a hollow tip is placed directly onto the surface of a neurone under the microscope in order to make real-time electrical recordings using the whole-cell patch-clamp method. An electrical circuit is set up that incorporates the neurone, microelectrode, current monitoring system and a specialised amplifier. Within the circuit, the resistance is fairly constant and the voltage may be 'clamped' at a specific point, thus changes to the current associated with ionic flux may be studied using Ohm's law ($I = V/R$). When an agonist binds to a synaptic receptor it induces a conformational change, which opens the channel allowing ions to travel across the membrane according to their electrochemical gradient. This process may be manipulated pharmacologically and the effects observed using electrophysiological techniques.

2.2 Breeding and housing of mice

The study was performed under the project licences of my supervisors and as such, has been subjected to rigorous ethical review. All procedures were carried out in compliance with the University of Dundee code of practice. Wild Type (WT) mice aged less than 2 months were obtained from an in-house colony. C57/ Bl6J Mice aged 2 months were purchased from Charles River, UK while OlaHSD *ob/ob* mice, *db/db* mice and their respective strain-matched WT littermates were purchased from Harlan UK and housed in the same in-house colony. Animals were kept under an alternating 12hr light/dark cycle and had *ad libitum* access to food and water. Prior to dissection mice were killed instantly by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986 (UK).

2.3 Spinal cord dissection technique

Spinal cord extraction from a mouse had not been performed within the host laboratory before and therefore this technique needed to be developed. This was achieved by reviewing the literature, by drawing upon the knowledge of a laboratory colleague who had previously worked with rat spinal cord slices in the sagittal plane and also by contacting colleagues in other research groups for advice. The relatively small size of the mice (particularly early in development) presented a considerable technical challenge. The removal of a spinal cord in good condition required a significant amount of training and experience. The two main techniques for obtaining the spinal cord in rodents are hydraulic extrusion and anterior laminectomy (Chery & De Koninck, 1999; Chery *et al*., 2000; Keller *et al*., 2001). After initial success with hydraulic extrusion the anterior laminectomy technique was adopted in an effort to improve reliability. Dr Gareth Miles (Principle Investigator) and Dr Catherine Dunford (former PhD student) of the University of St Andrews use the anterior laminectomy technique and a visit was made to their laboratory to observe their approach. After observing these methods I adapted their technique for use in my host laboratory.

Hydraulic extrusion

Mice were killed using the cervical dislocation method (Schedule 1) and then decapitated. The body was then submerged immediately in the prone position into a bath of ice-cold oxygenated (95% O_2 / 5% CO_2) artificial cerebrospinal fluid (aCSF) containing: 225mM sucrose, 10mM glucose, 10mM $MgSO_4$, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 2.5mM KCl, 0.5mM $CaCl_2$, 1mM ascorbic acid and 3mM pyruvic acid (total osmolarity ~330mosm/l). Scissors were used to remove a 1cm wide strip of skin to expose the fascia-covered dorsal aspect of the spinal column. Toothed forceps held the body, while fine scissors were used to extract the entire spinal column carefully from the carcass. The spines were palpated to identify the presence of any fractures of the (thoracic) vertebrae. Generally, fractures were associated with unsuccessful extrusion, but this problem could be minimised by trimming the column below the level of the fracture. A razor blade was used to cut through the spinal column perpendicularly at the mid-thoracic and sacral levels. Any blood and debris was washed off using a 10ml syringe and the caudal end of the cord was trimmed further as required using the razor blade. This approach allowed the cauda equina to be visualised within the sacral end of the spinal canal. A 21-gauge needle (connected to a 20ml syringe filled with sucrose solution) was then inserted into the spinal canal. Manual pressure was applied to the plunger, which generated hydraulic pressure within the spinal canal, forcing the spinal cord to be expelled into the ice-cold solution. The dura was then removed to prevent it impairing the process of slicing the spinal cord.

Anterior Laminectomy

Mice were killed by cervical dislocation (Schedule 1) and then decapitated. The body was immersed in ice-cold oxygenated, aCSF solution and after placing it supine, the limbs were extended and pinned to the floor of a dissection chamber, modelled on the vessel used by the Miles laboratory in St Andrews. [The base of a small oblong can (Herring in Mustard and Dill Sauce, *John West Ltd*) was filled with black Sylgard to a depth of 1-2 mm to enable the animal body to be pinned down

for dissection.] A gaseous mix of 95% O_2 / 5% CO_2 was bubbled into the chamber continuously and it was placed into a tray of ice to maintain the low temperature required to preserve the tissue.

Scissors (9cm, No. 14568-09, FST, Heidelberg, Germany) were used to cut away the skin to expose the thoracic cage. The ribs were then cut either side of the sternum to open the thoracic cavity. The internal organs were removed to reveal the anterior aspect of the vertebral column. Small toothed forceps grasped the most cephalad vertebral body at the cervical level and the fine scissors (No. 15006-09, FST, Heidelberg, Germany) were used to cut the vertebral arches immediately posterolateral to the vertebral bodies. By this method the vertebral bodies were detached consecutively to reveal an intact spinal cord. The cervical end of the cord was then grasped with the fine forceps and peeled out of the vertebral canal gently using fine scissors (No. 15008-08, FST, Heidelberg, Germany) to cut through the roots. The cord was pinned to the chamber and the remaining dura and nerve roots were removed to facilitate optimal slicing. Removal of the dura and nerve roots is a crucial step to facilitate the success of the preparation.

2.4 Spinal cord slice preparation

Initial technique: No supporting block

The cord was removed from the solution by grasping the caudal tip with fine forceps and then placed briefly onto moist filter paper to remove excess solution. The cord was then placed onto a 1cm cube of polystyrene/Sylgard/agar, which had previously been glued onto the metal disc. The flat thoracic end was held in place using a small amount of glue on the metal disc and by mild natural adherent properties to the cube. A Leica VT1000S vibratome (Heidelberg, Germany) was set to a forward speed of ~0.08mm/s and to the maximal side-to-side vibration speed of approx. 80Hz. Horizontal, thoracolumbar slices, (300-350μm) were cut and transferred onto a nylon mesh platform within a storage chamber containing oxygenated (95% O_2 / 5% CO_2) artificial extracellular solution (aECS) comprising: 126mM NaCl, 10mM glucose, 2mM $MgCl_2$, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4,

2.95mM KCl, 2mM $CaCl_2$, 1mM ascorbic acid, 3mM pyruvic acid and 2mM kynurenic acid (Total osmolarity ~310mOsm/l). Slices were stored at room temperature for at least one hour before being used to obtain electrophysiological recordings.

Definitive technique: Agar block

This technique was first observed in the Miles Group Laboratory, University of St Andrews and modified slightly to suit requirements. Agar powder was dissolved in aCSF solution at a concentration of approximately 1% and heated for approximately 20-30 seconds in a microwave oven in a glass beaker until just before it started to boil. The agar solution was allowed to cool to ~36^{o}C while being stirred continuously. The agar was poured into a small agar plate and the spinal cord placed into the gel itself before it set. After setting, a cuboid of agar containing the cord was then extracted from the plate. The agar cube was glued onto the vibratome platform with another cube of sylgard, or agar (10%) alongside for support. The agar provided support for the cord and typically released the small slices after slicing.

Brain slicing technique

Mice were killed using the cervical dislocation method (Schedule 1) and then decapitated. The head was then submerged immediately into a bath of ice-cold oxygenated (95% O_2 / 5% CO_2) aCSF. For all nRT preparations the aCSF solution was the same as that described above for spinal cord but the sucrose concentration was increased to 234mM giving a total osmolarity of ~340mOsm/l as this improved the condition of the slices. For cortical preparations in mice below 2 months of age the aCSF was the same as for the spinal cord. Neuronal viability deteriorates with increasing age and for mice above the age of 2 months, including all *ob/ob* and *db/db*, a different solution was required to optimise the condition of the slices. This consisted of 140mM potassium gluconate, 10mM HEPES, 15mM

sodium gluconate, 0.2mM EGTA, 4mM NaCl, 1mM ascorbic acid and 3mM pyruvic acid. Sodium hydroxide solution was then added to bring the pH up to 7.2. The brain was removed carefully from the skull and slices were obtained using a Vibratome series 1000 PLUS Sectioning System (Intracell, Royston, Hertfordshire, UK). Cortical slices were cut in the coronal plane and nRT slices in the horizontal plane. Slice thickness was greatest for the youngest mice and least for the oldest mice. For instance, at postnatal day 6 (P6) it was 350μM while at postnatal day 60 (P60) it was 250μM. Slices were transferred immediately to a storage chamber as previously described for the spinal cord.

Electrophysiology

Subsequently, the slices were transferred to a recording chamber under an Olympus BX51WI fixed-stage upright microscope. An infrared differential interference contrast disc and a water immersion objective (x 40) were used for visualisation of neurones within the spinal, or brain, tissue. The chamber was perfused continuously with oxygenated aECS. Slices were held in position using a small grid, fashioned using a flattened platinum wire and pieces of nylon tights. A magnification chamber (x 1.6) was also used to increase the magnification of the image further. Microscopic images were visualised on a video monitor to facilitate the experimental process.

Lamina II (LII) neurones of the dorsal horn of the spinal cord are typically oblong in shape. They were selected visually from the translucent band of neural tissue that lies between LI and LIII. The dorsal horn of the spinal cord is comprised of LI and LII and has an integral role in transmission of nociceptive impulses from the periphery to higher centres (D'Mello & Dickenson, 2008). Previous work has suggested that loss of fast synaptic inhibition mediated by the $GABA_AR$ located on LII neurones may be implicated in the development of allodynia and hyperalgesia (Keller *et al.,* 2004, Sivilotti & Woolf, 1994). In addition, the upregulation of endogenous neurosteroids in response to peripheral inflammation may mediate an endogenous analgesic effect (Poisbeau *et al.,* 2005).

Neurones of the nRT are also oblong in shape and form a distinct longitudinal band immediately lateral to the perpendicularly striated ventrobasal (VB) complex of the thalamus. This anatomical relationship facilitates visual identification. The VB complex relays somatosensory impulses from the spinal cord to higher centres. Neurones of the nRT mediate GABAergic inhibitory tone over VB neurones, thus providing physiological regulation of somatosensory transmission (Arcelli *et al.,* 1997; Clascá *et al.,* 2012; Cox *et al.,* 1997; Gentet & Ulrich, 2003; Huh, 2012).

Pyramidal neurones located in layer 2-3 of the cerebral cortex are easy to identify due to their distinctive shape comprising pyramid-shaped body with prominent apical dendrite. There is also a pronounced natural demarcation between layers 1 and 2 of the cortex. The former contains few cell bodies while the latter contains densely packed neurones (mostly pyramidal). Cortical neurones are organised into functional units comprising vertical columns from layer 2-6. These layers receive direct synaptic connections from the thalamus and layer 2-3 pyramidal neurones also provide intercortical GABAergic inhibitory modulation (Clascá *et al.,* 2012; DeFelipe & Farinas, 1992; Mountcastle, 1997).

Whole-cell patch-clamp recordings were obtained using an Axopatch 200B amplifier with up to 75% capacitance compensation. All recordings were made at a holding potential of -60mV and at a temperature of 35^{o}C. The extracellular solution contained kynurenic acid (2mM), strychnine (0.5μM) and tetrodotoxin (0.5μM) to antagonise ionotropic glutamate, glycine receptors and voltage-gated sodium channels respectively. Patch electrodes were prepared from thick walled borosilicate glass capillaries (Garner Glass Co., Claremont, CA, USA). Such electrodes had an open-tip resistance of ~4MΩ when filled with intracellular solution containing: 135mM CsCl, 10mM HEPES, 10mM EGTA, 1mM $CaCl_2$, 1mM $MgCl_2$, 2mM Mg-ATP, 5mM QX-314, pH approx 7.2. The osmolarity was approximately 310mOsmol/l. Typically, the mean whole-cell capacitance was 5-15pF and only recordings where the series resistance changed by less than 20% were analysed. A 2 kHz frequency filter was used for all recordings and analysis of each cell was performed offline as described below.

2.5 Data analysis

The Strathclyde Electrophysiology Software, WinEDR and WinWCP, (Dr J Dempster, University of Strathclyde, Glasgow, UK) was used for the analysis of recordings. Only recordings that met specific quality criteria were included in the analysis. mIPSCs were detected with an amplitude threshold of at least -5pA and duration > 2ms. Each individual mIPSC was then inspected visually to ensure validity and exclude artifactual events. mIPSCs with a rise time of more than 1ms were excluded to prevent the inclusion of events originating from a distal source. At least 50 events were sought for each recording, and their peak amplitude, rise time (10-90%), charge transfer (area under the curve) and T_{70} (time required to decay by 70%), were analysed. The mIPSCs were digitally averaged by alignment at the midpoint of the rising phase. The mIPSC decay was fitted by monexponential ($y(t) = Ae^{-t/\tau}$) and biexponential ($y(t) = A_{fast}e^{(-t/\tau fast)} + A_{slow}e^{(-t/\tau slow)}$) functions to determine which one was more appropriate. (A is amplitude, t is time and τ is the decay time constant; Figure 6).

The mIPSC exponential decay time τ_w (weighted Tau), is a mathematical constant generated by considering the fast initial component of biexponential decay τ_1 and the later slower component τ_2. The value of τ_w is determined for the mean mIPSC for each cell by determining the relative proportion that τ_1 and τ_2 contribute to the biexponetial decay. The following equation was used: $\tau_w = \tau_{fast}P_1 + \tau_{slow}P_2$, where P_1 and P_2 represent the proportions of the synaptic current decay curve described by each component (Figure 7).

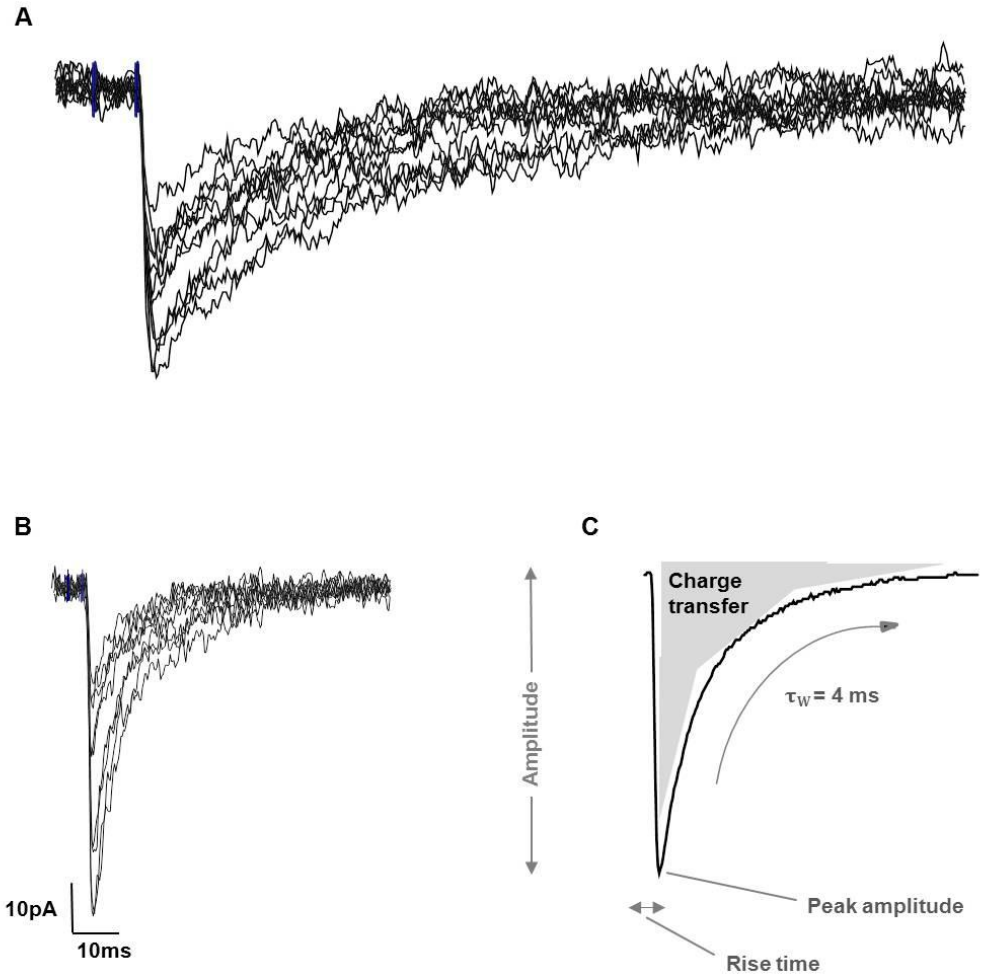

Figure 6 Superimposed individual mIPSCs from spinal & cortical neurones.

(A) Superimposed individual mIPSCs from a single spinal neurone showing intra-cell variation in the peak amplitude. Note the 'noisy' appearance of individual mIPSCs. **(B)** Superimposed individual mIPSCs from a single cortical neurone. **(C)** An averaged cortical mIPSC composed of 172 individual events extracted from one representative (or exemplar) recording. Note the rise time, peak amplitude and the decay time constant τ_w.

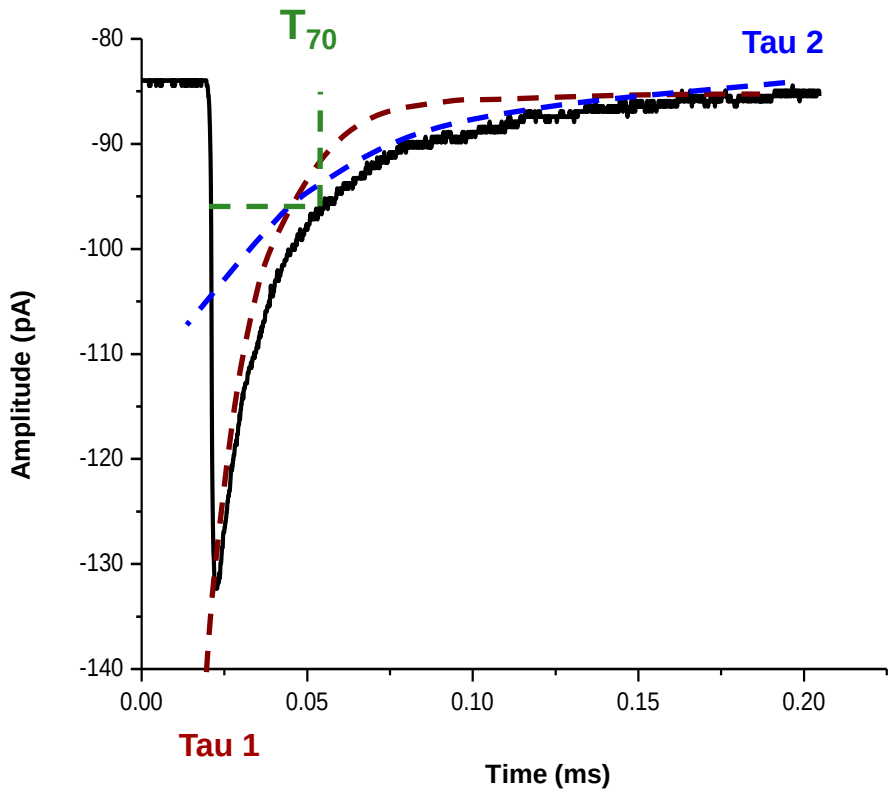

Figure 7 Determination of GABA$_A$R mIPSC decay time constant.

mIPSCs were digitally averaged by alignment at the midpoint of the rising phase. The mIPSC decay was fitted by monexponential ($y(t) = Ae^{-t/\tau}$) and biexponential ($y(t) = A_{fast}e^{(-t/\tau fast)} + A_{slow}e^{(-t/\tau slow)}$) functions to determine which one was more appropriate. (A is amplitude, t is time and τ is the decay time constant). The mIPSC exponential decay time τ_w (weighted Tau), is a mathematical constant generated by considering the fast initial component of biexponential decay τ_1 and the later slower component τ_2. The value of τ_w is determined for the mean mIPSC for each cell by determining the relative proportion that τ_1 and τ_2 contribute to the biexponetial decay. The following equation was used: $\tau_w = \tau_{fast}P_1 + \tau_{slow}P_2$, where P_1 and P_2 represent the proportions of the synaptic current decay curve described by each component.

The signal-to-noise ratio prevents the meaningful fitting of time constants to individual mIPSCs. To investigate the kinetics of individual mIPSCs the parameter of T_{70} was determined. This parameter can be readily determined for individual mIPSCs and was chosen rather than the the T_{90}, which is subject to imprecision due to artefact, or fluctuations, in the tail of the synaptic event.

The tonic current was analysed with the same software package Win EDR utilised for the analysis of the synaptic events (Dr J Dempster, University of Strathclyde, Glasgow, UK). The baseline current and noise were sampled every 51.2ms over a 60 second period for each section analysed. At the sampling rate of 10kHz, one data point consisted of 512 baseline points. Recordings were scrutinised in order to remove mIPSCs and artefacts. Recordings made pre- and post- drug application were compared to determine if there were significant changes to the baseline holding current. In some recordings, the baseline holding current can drift spontaneously over time. Only baseline shifts that were twice the standard deviation of the control section were considered genuine effects. This provided a systematic and reproducible way of determining if a shift in the baseline current in response to an applied drug was genuine, or not.

The frequency of mIPSCs was also analysed using the WinEDR software. An event detection protocol was initiated with 1ms dead time over for at least 40 seconds. Two separate sections of recording were analysed and the mean value of the two sections combined was used to derive the frequency for an individual neurone. Each section was inspected carefully to remove spurious ‘hits’ and to insert any missed events. If an individual event was contaminated by a second event, then both events were counted (in contrast to the analysis of mIPSC decay time, when these events were omitted).

2.6 Drug and solution preparation and administration

The numerous salts used in the preparation of aECS and aCSF solutions were purchased from VWR (West Chester, Pennsylvania, USA). Strychnine (Sigma Chemicals, St. Louis, MO, USA), tetrodotoxin (Tocris, Bristol, UK) and bicuculline (Axxora, Nottingham, UK) and THIP (generous gift from B Ebert) were prepared as concentrated stock solutions in double-filtered water to be added to the aECS. Other compounds such as progesterone, ganaxolone, allopregnanolone, dihydroxy-progesterone, provera, indometacin, finasteride, diazepam and flumazenil were (purchased from Tocris or Sigma) and prepared as concentrated stock solutions in DMSO. The cyclodextrins (Sigma) were dissolved directly into the aECS, or the intracellular solution, as outlined in the results section.

Brain and spinal cord slices were placed under the microscope into a transparent plastic chamber filled with aECS. The aECS was perfused through the chamber using a rate-adjustable gravity-based system consisting of plastic tubing that connected an oxygenated reservoir to the chamber. Simultaneously, a peristaltic pump system (Minipuls 3, Gilson, UK) drained the aECS from the opposite side of the chamber and recycled it back to the reservoir. The fluid-filled circuit also ran through a custom made heating system (G23, UCL, London, UK) controlled by a temperature monitoring system (School of Pharmacology, London, UK) that maintained the near physiological temperature of 35^{o}C throughout the course of every experiment (physiological temperature= 36.5-37.5^{o}C).

2.7 Behavioural experiments

Drug adminstration

The drug, or vehicle, was administered by intra-peritoneal injection using a fine 1ml syringe and tail flick recordings were made pre- and post- injection. Neurosteroids are lipophilic compounds that have very limited solubility in aqueous solution, but can be solubilised in 0.9% saline using 2-hydroxy propyl β-

cyclodextrin to facilitate administration (Besheer *et al.,* 2010; Carter *et al.,* 1997; Reddy & Rogawski, 2010). Neurosteroids were therefore administered in 40% β-cyclodextrin solution.

Rotarod test

The rotarod test comprises an elevated rotating cylinder upon which a rodent is placed (Jones & Roberts, 1968; Pritchett & Mulder, 2003). In order to avoid falling off the rotarod, the mouse must maintain constant motion; hence it is a test of forced motor activity (Jones & Roberts, 1968; Pritchett & Mulder, 2003). To remain on the rotarod while it accelerates at a set rate the mouse requires balance and coordination. The rotarod may be used to test motor function after the administration of novel agents as part of the drug development process, or to characterise neurodegenerative conditions such as Huntington's disease and Parkinson's disease (Pallier *et al.,* 2009; Shiotsuki *et al.,* 2010). Mice were placed on the rotarod and the accelerating rotarod protocol was used. Specifically, the rod starts to rotate at 6 revolutions per minute (rpm) and is then increased in 4 rpm increments up to a maximum of 50 rpm. The experiment continues until the mouse falls off, or until the cut-off time of 300 seconds has elapsed.

Thermal nociception

The tail flick test was first described in 1941 as a simple and reproducible measure of thermal pain and has become a standard behavioural test in rodents. In the initial description, heat from a light bulb was focused directly onto the tail of a rat and the latency time until the tail was flicked away was recorded with a stopwatch (D'Armour & Smith, 1941, Mogil *et al.,* 2009). For the purposes of the experiments described here a modified tail flick test was employed. Thermal nociceptive thresholds were assessed by the immersion of 2cm of the animal's tail in a water bath maintained at a specified temperature such as 40-50 ^{o}C until the tail flick manoeuvre was initiated. The tail flick latency time was recorded and this parameter was used to compare the effect of neurosteroids *versus* injection of

control vehicle and baseline measurements. A cut off time of 15 seconds was used to minimise the likelihood of tissue damage. The results of each experiment may be expressed in seconds, or as a percentage of the maximum possible effect (MPE) *i.e*. a percentage of 15 seconds.

Mechanical nociception

In the late nineteenth century, Max von Frey explored sensory thresholds using a variety of objects including tough horsehair. His ideas were developed by others and nylon von Frey filaments are now used routinely in human and animal research (Mogil *et al.,* 2009, Pearce 2006). A series of calibrated von Frey filaments (Ugo Basile, It) were employed to characterise mechanical nociceptive thresholds in WT and *ob/ob* mice. The mice were placed into clear plastic cubicles on top of a raised platform (Ugo Basile, Italy) with a meshed surface and allowed to acclimatise to the new environment for 30 minutes. The tip of the von Frey filament was pressed carefully onto the middle of the ventral ('palmar') surface of the hindpaw. Sufficient force to induce bending of the shaft of the filament was applied for up to 5 seconds and the presence of a withdrawal response noted if it occurred. The procedure was repeated until each hindpaw had received 5 presses of the filament. Only robust and immediate withdrawal responses were considered as positive. Each mouse could have a maximum score of 10 (five for each paw). Testing would commence with the thinnest filament used and then progress to thicker filaments. Pilot studies were carried out to determine the optimal four filaments to be used in the mice; 0.16g, 0.4g, 0.6g and 1g filaments. They elicited a response in approximately 20%, 40%, 60% and 90% of occasions when applied to adult WT mice. This meant that these filaments could be used to test for the presence of mechanical allodynia, hyperalgesia and nociceptive pain in the *ob/ob* mouse. This method was adapted from work published in rats with neuropathic sensitisation (Meyer *et al.,* 2011).

Cold allodynia test

The sensing of a wide range of environmental temperatures is a useful physiological process that confers survival advantages in many species. Indeed, adapting to a cold external environment can be as important as withdrawing from a burning hot stimulus. The thermal gated ion channels TRPM8 and TRPA1 appear to be important to the generation of an afferent neural impulse in response to a cold stimulus in the noxious range. Increased sensitivity to cold stimuli is a common symptom associated with neural injury and although the underlying mechanisms responsible for this phenomenon are poorly understood it may be related to increased expression of TRPM8 and TRPA1 channels (Belmonte *et al.,* 2009; Knowlton *et al.,* 2010). Conversely, knockout of TRPM8 in mice results in a significantly reduced response to cold stimuli such as topical acetone (Belmonte *et al.,* 2009). When acetone is applied topically to the skin it evaporates and induces a cold sensation. When neural hypersensitivity is present acetone may induce a temporary noxious stimulus. Acetone has therefore been used to elicit cold allodynia in animals with chemotherapy-induced neuropathic hypersensitivity (Flatters & Bennett, 2004, Gauchan *et al.,* 2009, Meyer *et al.,* 2011).

Mice were placed into the same apparatus as described previously in the mechanical nociception section. A drop of acetone was applied to the ventral (palmar) surface of the animal's hindpaw using a modified 1ml syringe. A standardised scoring procedure was used to record the effect of acetone application with slight modification of that described previously in the literature (Flatters & Bennett, 2004). After the application of acetone, the mouse was observed for 20 seconds and if a response was elicited, the animal was observed for an additional 20 seconds in order to ensure that pain related behaviour was evoked rather than merely a startle response. The process was performed twice on each hindpaw after an interval period. In the event that it was felt that the drop had missed the paw it was reapplied immediately.

The following scoring system was used:

0 No response

1 Quick withdrawal, flick or stamp of the paw only

2 Prolonged/repeated flicking of the paw and/or abnormal hindlimb posture/limping

3 Same as (2) but with additional licking of the ventral surface of the affected paw

2.8 Statistical analysis

All data in the results section are expressed as the arithmetic mean ± the standard deviation (SD). The following statistical tests were employed where appropriate for the electrophysiological data: Student's t test (Excel, Microsoft Office), One-way ANOVA, One-way and Two-way RM ANOVA (Sigmastat) and the Kilmogovov Smirnoff test (SPSS software, IL, USA). The following non-parametric statistical tests were employed for the behavioural data: Mann-Whitney Rank Sum test, Kruskal Wallis one-way ANOVA on ranks and the Wilcoxon signed rank test (before & after; Sigmastat).

Chapter 3: A characterisation of GABA-ergic synaptic events with development at three levels of the pain pathway in WT mice.

Inhibitory synaptic transmission innervating neurones at three levels of the pain pathway was studied using the whole-cell patch-clamp electrophysiological technique. Neuronal recordings were made from C57/Bl6 mice in lamina II (LII) of the dorsal horn of the spinal cord, the nucleus reticularis (nRT) of the thalamus and layer 2/3 of the cerebral cortex (See Figure 8). These neurones were selected for study due to their modulatory role in nociceptive transmission, associated with the perception of pain in humans (Clascá *et al.,* 2012; Cox *et al.,* 1997; DeFelipe & Farinas 1992; D'Mello & Dickenson 2008; Gentet & Ulrich 2003). Previous work in the host laboratory (Brown, 2012) on mouse neurones of the cortex and thalamocortical neurones of the ventrobasal thalamus revealed that fluctuations in the endogenous neurosteroid tone during development (P7–P24) influenced the duration of miniature inhibitory postsynaptic currents (mIPSCs). Such events arise from the activation of synaptic $GABA_A$Rs by synaptic GABA released from a single vesicle (as described in Part 2 of the Introduction). Previous studies have proposed that similar changes to the properties of spinal LII mIPSCs may occur with development in rats (Inquimbert *et al*., 2007; Keller *et al*., 2001, Keller *et al*., 2004; Poisbeau *et al*., 2005) and mice (Rajalu *et al*., 2009).

3.1 The decay time of $GABA_A$R mIPSCs of LII spinal neurones decreases with development.

Whole-cell voltage-clamp recordings from LII neurones of the dorsal horn were made for C57/BL6 mice of both sexes from three post-natal age groups: 8-11 days, 17-25 days and 60-75 days. Analysis of phasic inhibition (as described in the Methods section) included the amplitude, the decay kinetics, the charge transfer, the frequency and the rise time of mIPSCs recorded from lamina II neurones of the mouse spinal cord. The mean peak amplitude of LII mIPSCs for the three age groups studied here (P8-11 = -45 $\pm$ 4 pA, n = 26 neurones; P17-25 = -43 $\pm$ 2.7 pA, n = 31 neurones; P60 = -41 $\pm$ 4.6 pA, n = 13 neurones) did not differ significantly with development (One way ANOVA, $P > 0.05$; Table 1).

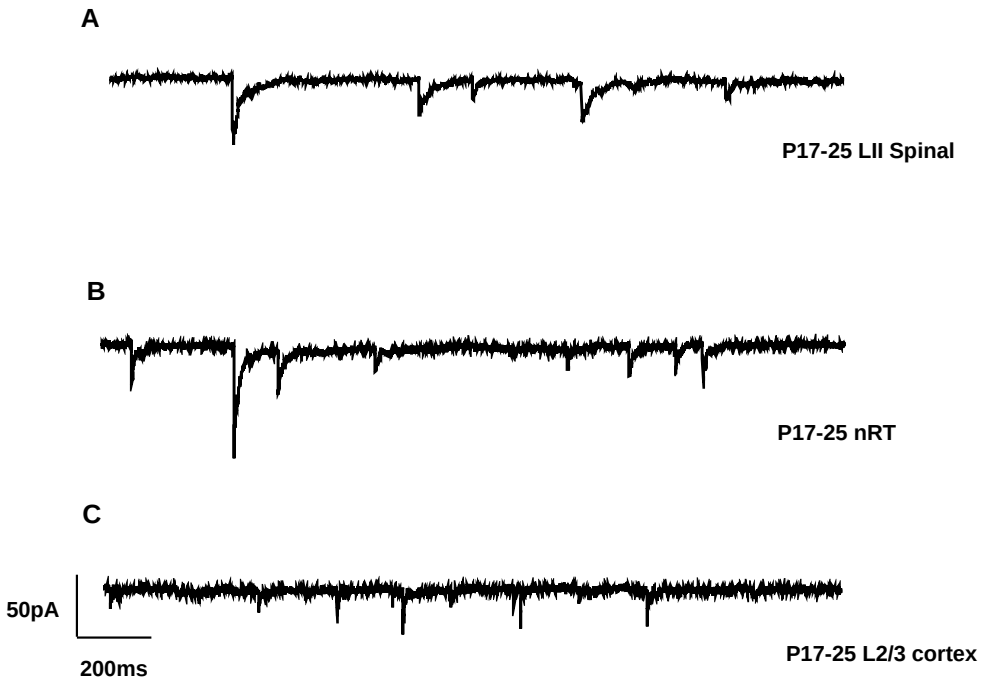

Figure 8 Representative current traces from neurones aged P17-25 exhibiting $GABA_AR$ mIPSCs from three levels of the pain pathway illustrating the regional variations.

(A) A two second section of current recording from a representative LII spinal neurone voltage clamped at -60mV and recorded in the presence of 0.5μM TTX, 0.5μM strychnine and 2mM kynurenic acid. The downward deflections are $GABA_AR$ mIPSCs. **(B** & **C)** Current recordings from representative nRT and L2/3 cortical neurones aged P17-25 under the same conditions. Note that mIPSCs from nRT and LII neurones have a similar appearance, while the cortical mIPSCs have a much shorter duration.

Table 1 The decay time of $GABA_AR$ mIPSCs of LII spinal neurones decreases with development

	P8-11 (n=26)	**P17-25 (n=31)**	**P60-75 (n=13)**
Peak amplitude (pA)	-45 ± 4	-43 ± 3	-41 ± 5
Rise Time (ms)	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	**-965 ± 82	-821 ± 89	-652 ± 80
T70 (ms)	****26.5 ± 3**	****19.6 ± 2**	**17.6 ± 2**
Tau w (ms)	****24.8 ± 2**	****19.4 ± 2**	**17.5 ± 2**
Frequency (Hz)	1.1 ± 0.2	0.6 ± 0.1	0.7 ± 0.2

***P* < 0.05; One-way ANOVA, statistically significant in comparison to P60-75

n = number of neurones

Previous studies of LII neurones revealed the mIPSC time course of decay may reduce with development, a perturbation that may be caused by the loss of an endogenous steroid tone (Keller *et al*., 2001, Keller *et al*., 2004, Rajalu *et al.,* 2009). The mIPSC decay (τ_W) decreased with development (P8-11 = 24.8 ± 2 ms; n = 26; P17-25 = 19.4 ± 1.8 ms, n = 31; P60-75 = 17.5 ± 1.8 ms, n = 13; One-way ANOVA, *P* <0.05 - Table 1; Figure 9). The τ_W is approximately equivalent to the time taken for the mIPSC to decrease by 67% from the peak amplitude. The unfavourable signal-to-noise of individual mIPSCs precludes the accurate fitting of the τ_W to individual mIPSCs. Therefore, this function is fitted to the mean mIPSC derived for each neurone *i.e.* a comparison of the 'mean of the mean'. Consequently, subtle differences may be obscured when making comparisons between different age groups. The T_{70} (time taken for the mIPSC to decay by 70% from the peak amplitude) is readily derived for individual mIPSCs. This parameter also changed significantly with development: (P8-11 = 26.5 ± 2.7 ms; n = 26; P17-25 = 19.6 ± 1.8 ms, n =31, P60-75 = 17.6 ms ± 1.8 ms, n = 13 (One-way ANOVA, *P* <0.05; Table 1).

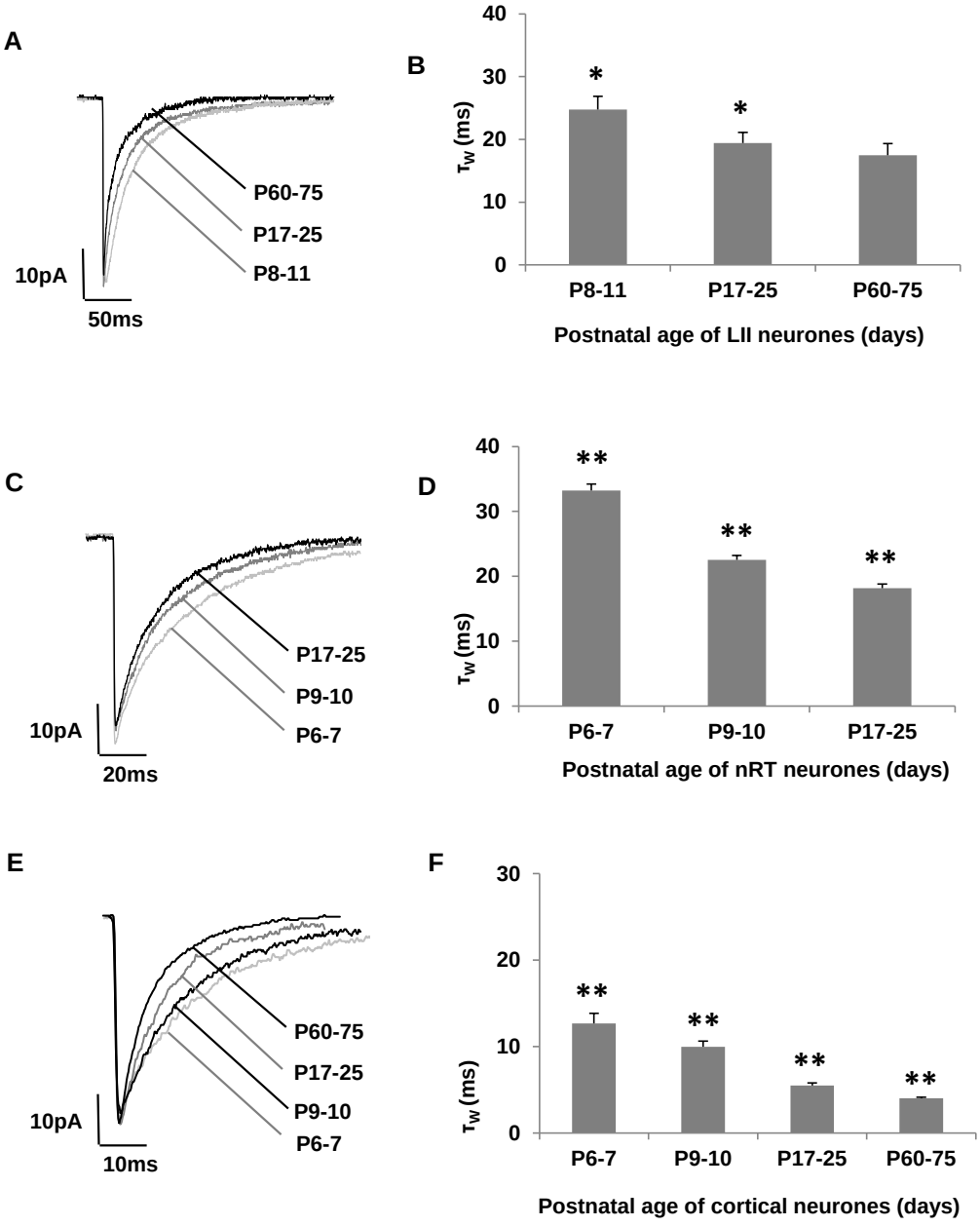

Figure 9 The decay time of $GABA_AR$ mIPSCs of neurones from three levels of the pain pathway decreases with development.

(A) Superimposed exemplar $GABA_AR$-mediated mIPSCs recorded from representative spinal neurones from three stages of development: P8-11 (light grey), P17-25 (grey) and P60-75 (black). Note the reduction of $GABA_AR$ mIPSC decay time that occurs with development. **(B)** Histogram illustrating the shortening of $GABA_AR$ mIPSC decay time (τ_W) with development (P8-11 = 24.8 ± 2 ms; P17-25 = 19.4 ± 1.8 ms; P60-75 = 17.5 ± 1.8 ms; One-way ANOVA $^*P < 0.05$; n = 13-31). **(C** & **D)** A parallel developmental change is observed in nRT neurones (P6-7 = 33.2 ± 1 ms; P9-10 = 22.5 ± 0.7 ms; P17-25 = 19.6 ± 0.7 ms; One-way ANOVA $P < 0.05$. *Post hoc* Newman Keul's test revealed significant differences between all groups, $^{**}P < 0.05$; n = 14-24). **(E** & **F)** A parallel developmental change is observed in L2/3 cortical neurones (P6-7 = 12.7 ± 1.2ms; P9-10 = 10 ± 0.7ms; P17-25 = 5.5 ± 0.4ms, n = 8; P60-75 = 4 ± 0.1ms; One-way ANOVA $P < 0.05$. *Post hoc* Newman Keul's test revealed significant differences between all groups, $^{**}P < 0.05$; n = 7-35).

Irrespective of the mechanism, these data suggest that the majority of these developmental changes to inhibitory synaptic transmission in LII neurones have occurred by the age of ~17 days. There was no significant difference in the T_{70} between the immature male and female mice (spinal LII P8-11: female = 26.1 ± 3.8 ms, male = 27.1 ± 3.7 ms; spinal LII P17-25: female = 18.6 ± 1.7 ms, male = 21 ± 3.5 ms; Student's unpaired t tests, P >0.05). These findings are consistent with previous work performed on rats by a former colleague (Mitchell *et al*., 2007; Table 2). The parameter of mIPSC charge transfer (*i.e.* the mIPSC area) is influenced by changes to kinetics and amplitude and is readily determined for each mIPSC. It also changed significantly with development: (P8-11 = 965 ± 82 fC; P17-25 = 821 ± 89 fC; P60-75 = 652 ± 80 fC; One-way ANOVA, P <0.05; Table 1). The frequency of mIPSCs is influenced by the probability of vesicular release, which is determined by presynaptic neuronal function and modulation and factors such as the number of inhibitory synapses *per* LII neurone (Farrant & Nusser, 2005). Taken as a whole, these results are consistent with previously published data (Inquimbert *et al*., 2007; Keller *et al*., 2001, Keller *et al*., 2004; Poisbeau *et al*., 2005; Rajalu *et al*., 2009).

Table 2 The properties of $GABA_AR$-mediated mIPSCs in mice (P17-25) versus data recorded by a former laboratory colleague in rats (P15-21; Mitchell *et al.,* 2007).

	Mice P17-25 (n=31)	**Rats P15-21 (n=77)**
Peak amplitude (pA)	-43 ± 3	*-68 ± 3
Rise Time (ms)	0.6 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-821 ± 89	*-1258 ± 61
T70 (ms)	**19.4 ± 2**	**N/A**
Tau w (ms)	**19.6 ± 2**	***17.2 ± 0.6**
Frequency (Hz)	0.6 ± 0.1	*1.6 ± 0.1

*P < 0.05; unpaired Student's t test in comparison to mice

3.2 Glycinergic mIPSCs of lamina II neurones of the dorsal horn of the spinal cord.

The glycine receptor (GlyR) is an important mediator of neural inhibition within the spinal cord (Baer *et al.,* 2009; Lynch, 2009). Inhibitory glycinergic synaptic transmission innervating LII neurones within the dorsal horn of the mouse spinal cord was also studied using the whole-cell patch-clamp electrophysiological technique. There is a significant heterogeneity of $GABA_AR$ mIPSC decay kinetics within lamina II neurones of rats, but the decay kinetics of strychnine-sensitive glycine receptor mediated mIPSCs are much more homogeneous (Mitchell *et al.,* 2007). Here, both of these findings were replicated for dorsal horn neurones of mice aged 17-25 days (Figure 10; Figure 11). I have presented my data from mouse LII neurones here alongside published data on rat LII neurones from the host laboratory for comparison (Mitchell *et al.,* 2007). Note that rats reach maturity at a later age than mice and also that the rats studied were aged 15-21 days. The following data for GlyR mIPSCs is summarised in Table 3 alongside the rat data for comparison.

Table 3 The properties of glycine receptor mediated mIPSCs in LII spinal neurones of mice (P17-25) versus data recorded by a former laboratory colleague in rats (P15-21; Mitchell *et al.,* 2007).

	Mice P17-25 (n=23)	**Rats P15-21 (n=77)**
Peak amplitude (pA)	-93 ± 10	*-121 ± 6
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-321 ± 40	*-474 ± 27
T70 (ms)	**3.9 ± 0.3**	**N/A**
Tau w (ms)	**3.1 ± 0.2**	**3.1 ± 0.1**
Frequency (Hz)	1.1 ± 0.3	*2.4 ± 0.3

*$P < 0.05$; unpaired Student's t test in comparison to mice

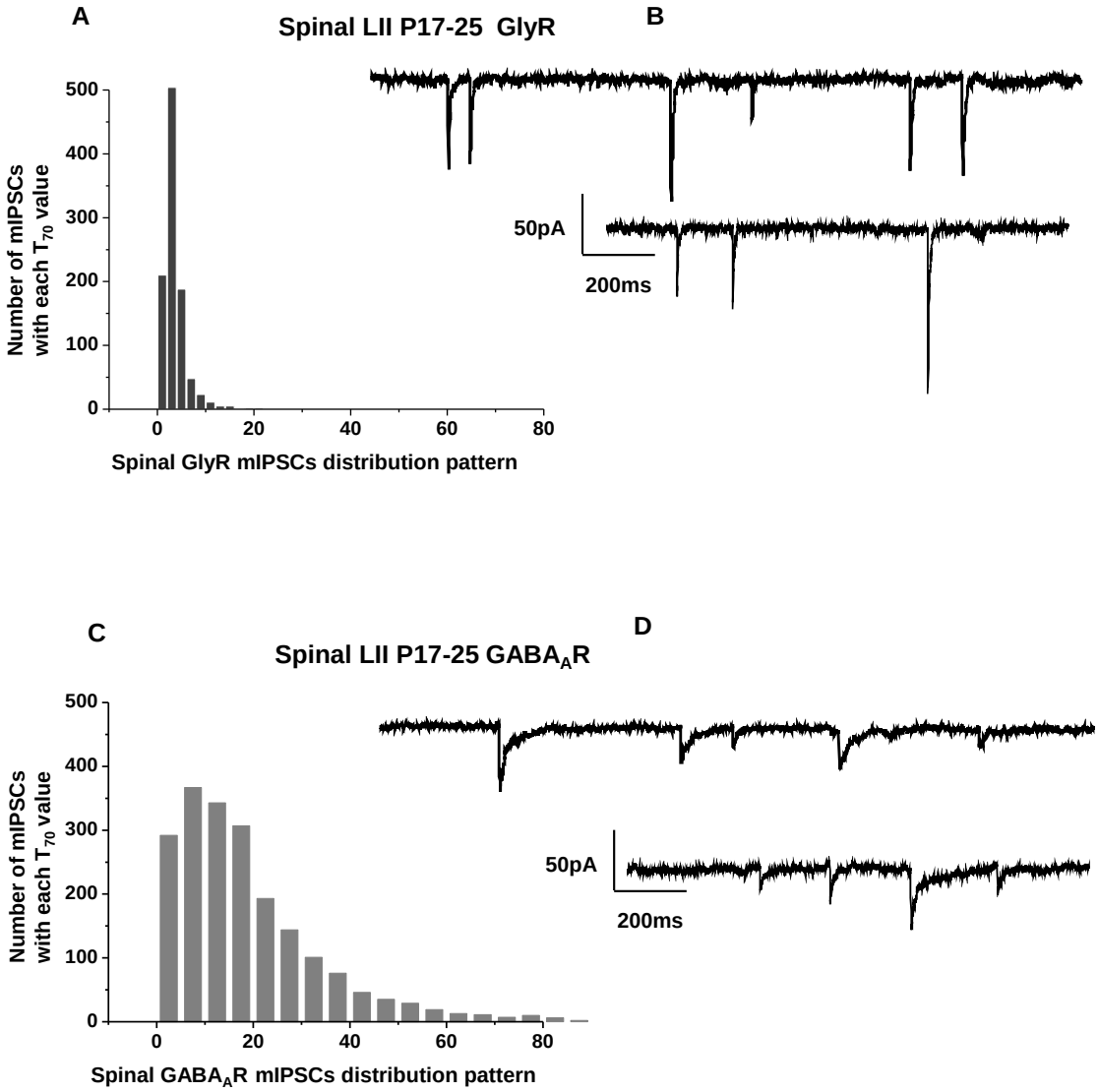

Figure 10 Representative current traces from LII spinal neurones aged P17-25 exhibiting $GABA_AR$ & GlyR mIPSCs.

(A) Histogram of the distribution pattern of T_{70} values of spinal GlyR mIPSCs indicating the relatively homogenous exponential decay times of synaptic glycinergic events. **(B)** A two second section of current recording from a representative LII spinal neurone voltage clamped at -60mV and recorded in the presence of 0.5μM TTX, 0.5μM strychnine and 30μM bicuculline. The downward deflections are GlyR mIPSCs. **(C)** Histogram of the distribution pattern of T_{70} values of spinal $GABA_AR$ mIPSCs indicating the relatively heterogenous exponential decay times of synaptic GABAergic events. **(D)** A two second section of current recording from a representative LII spinal neurone voltage clamped at -60mV and recorded in the presence of 0.5μM TTX and 2mM kynurenic acid. The downward deflections are $GABA_AR$ mIPSCs.

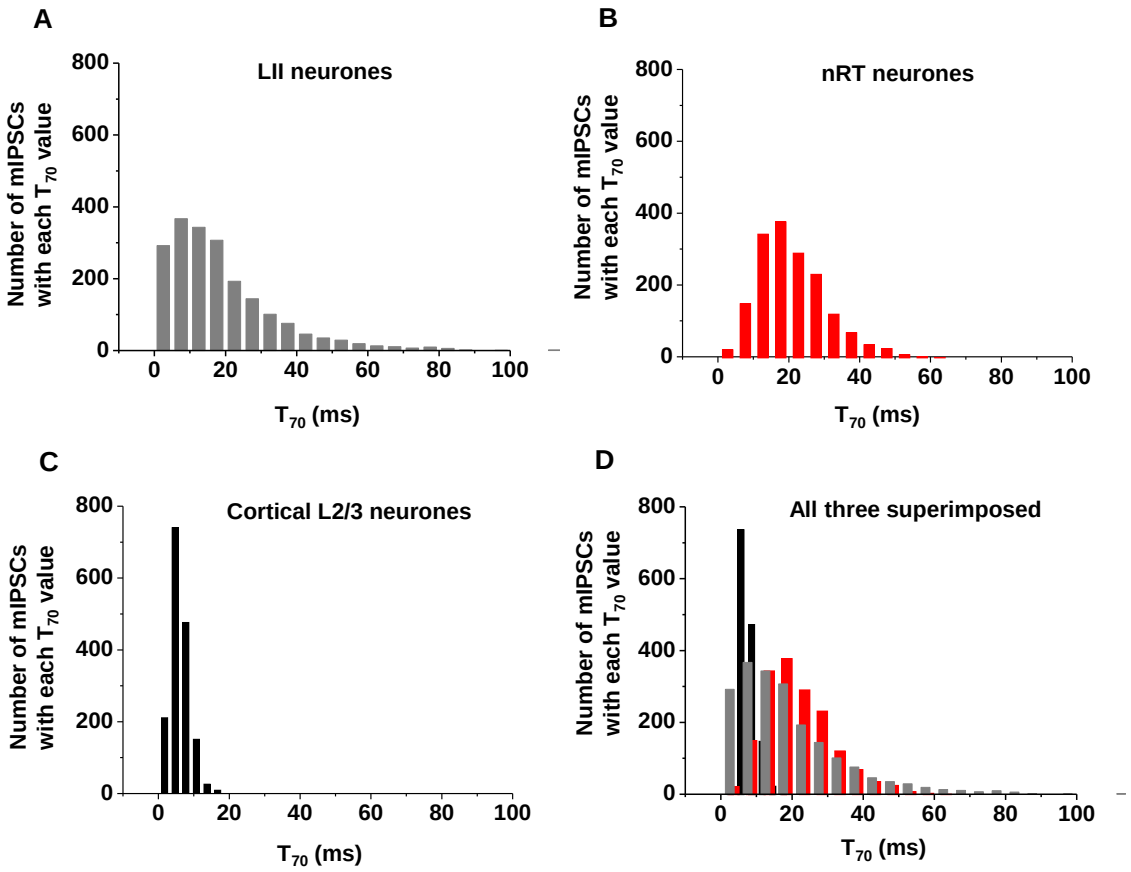

Figure 11 The distribution pattern of T_{70} values of $GABA_AR$ mIPSCs varies in neurones from three different levels of the pain pathway.

(A) Histogram of the distribution pattern of T_{70} values of spinal $GABA_AR$ mIPSCs indicating the relatively heterogenous exponential decay times of synaptic GABAergic events in these neurones. **(B)** Histogram of the distribution pattern of T_{70} values of nRT $GABA_AR$ mIPSCs indicating the more homogenous exponential decay times of synaptic GABAergic events. **(C)** Histogram of the distribution pattern of T_{70} values of L2/3 cortical $GABA_AR$ mIPSCs indicating the relatively homogenous exponential decay times of synaptic GABAergic events in these neurones. **(D)** Histogram of the distribution pattern of T_{70} values of $GABA_AR$ mIPSCs from three levels of the pain pathway superimposed to illustrate the differing distribution patterns of $GABA_AR$ mIPSC decay time.

The mean peak amplitudes of glycine receptor mIPSCs (P17-25 mice = -92.6 ± 10pA, n = 23) were smaller than in rats. The mIPSC rise time of glycine receptor mIPSCs was similar for both groups (P17-25 mice = 0.4 ± 0.1 ms, n = 23). The mIPSC decay (τ_W) of glycine receptor mIPSCs was also very similar for both groups (P17-25 mice = 3.1 ± 0.2 ms, n = 23). The T_{70} (time taken for the mIPSC to decay to 30% of peak amplitude) for P17-25 mice was 3.9 ± 0.3ms (no value available for rats). The parameter of mIPSC charge transfer was different between the two types (P17-25 mice = -321 ± 40fC, n = 23; P15-21 rats = 474 ± 27fC, n = 77). The frequency of mIPSCs was also different for the two species (P17-25 mice = 1.1 ± 0.3 Hz, n = 20; P15-21 rats = 2.4 ± 0.3 Hz, n = 77) but this may be related to differences in the technique used to slice the spinal cord tissue. The larger rat spinal cord was sliced in a parasagittal plane, while the smaller mouse spinal cord was embedded in agar and then sliced perpendicularly, in the horizontal plane. This is relevant because the synaptic projections run longitudinally in the spinal cord and more of these may be preserved with the parasagittal technique. Therefore, the frequency of synaptic events may be higher in the rat. In summary, there were only minor differences in the synaptic GABAergic and glycinergic data obtained from mouse LII neurones in comparison to an equivalent study performed with rat LII neurones.

3.3 The decay time of synaptic $GABA_A$Rs of nucleus reticularis (nRT) neurones decreases with development.

The nRT is the main source of GABAergic input into the thalamus and there are reciprocal GABAergic loops of innervation between nRT neurones and those of the VB. The two types of neurones, nRT and VB; regulate each other's function by this mutual inhibitory mechanism (Arcelli *et al.,* 1997; Cox *et al.,* 1997; Gentet & Ulrich, 2003; Guillery & Harting, 2003; Huh *et al.,* 2012). The inter-relationship between the nRT and VB acts to modulate nociceptive transmission and can therefore ultimately influence nociceptive behaviour in live animals (Huh *et al.,* 2012). Whole-cell voltage-clamp recordings from nRT neurones were made from C57/BL6 mice at three developmental stages: P6-7, P9-10 and P17-25.

Unfortunately, it is not practical to make such recordings from mice over the age of P25 due to the high density of axonal projections (Cox *et al.,* 1997; Pinault & Deschenes, 1998). Analysis of phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency. The analysis revealed changes to all of these parameters with development as detailed in Table 4. However, the principle focus here is on the decay kinetics of nRT GABA mediated mIPSCs. The mIPSC decay (τ_W) of nRT neurones decreased significantly with development (P6-7 = 33.2 ± 1 ms, n = 24; P9-10 = 22.5 ± 0.7 ms, n = 14; P17-25 = 18.2 ± 0.6 ms, n = 32; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between all groups, $P < 0.05$; Table 4; Figure 9). As expected, in common with the τ_W, the mIPSC T_{70} was markedly different at the three stages of development (P6-7= 35.8 ± 1 ms, n= 24; P9-10 = 25.4 ± 0.8 ms, n = 14; P17-25 = 20.1 ± 0.6ms, n = 32; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between all groups, $P < 0.05$; Table 4). These results demonstrate that in common with LII spinal neurones, the $GABA_AR$ mIPSC decay time of nRT neurones becomes significantly shorter during development (P6–P25).

Table 4 The decay time of $GABA_AR$ mIPSCs of nRT neurones decreases with development

	P6-7 (n=24)	**P9-10 (n=14)**	**P17-25 (n=32)**
Peak amplitude (pA)	**-56 ± 3	**54 ± 2	-46 ± 2
Rise Time (ms)	**0.7 ± 0.1	**0.7 ± 0.1	0.6 ± 0.1
Charge transfer (fC)	**-1475 ± 83	**-1089 ± 51	-750 ± 28
T70 (ms)	**35.8 ± 1	**25.4 ± 0.8	**20.1 ± 0.6**
Tau w (ms)	**33.2 ± 1	**22.5 ± 0.7	**18.2 ± 0.6**
Frequency (Hz)	*2.2 ± 0.4	6.9 ± 0.7	8.4 ± 1

***P* < 0.05; One-way ANOVA, in comparison to P17-25

3.4 The decay time of synaptic $GABA_A$Rs of cortical layer 2/3 neurones decreases with development.

GABAergic inhibitory neurones are present in all layers of the cortex but are most abundant in layers 2/3 and lower in layer 4 (Meyer *et al.,* 2011). They project vertically down to the deeper layers, exerting GABAergic inhibitory tone on pyramidal cells within the column (Helmstaedter *et al.,* 2009; Mountcastle, 1997). Electrophysiological recordings were made from cortical layer 2/3 neurones from C57/BL6 mice at four stages of development: P6-7, P9-10, P17-25 and P60-75. Analysis of phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency. The analysis revealed changes to all of these parameters with development as detailed in Table 5. However, here the principle focus again is on the decay kinetics of cortical GABA mediated mIPSCs. Previous studies of spinal neurones have revealed that the mIPSC decay time course decreases with development, a perturbation that may be caused by the loss of an endogenous steroid tone (Keller *et al*., 2001, Keller *et al*., 2004, Rajalu *et al.,* 2009). Work by a former laboratory colleague in cortical layer 2/3 pyramidal neurones (P7 - P24) is also consistent with these observations (Brown, 2012).

Table 5 The decay time of $GABA_A$R mIPSCs of layer 2/3 cortical neurones decreases with development

	P6-7 (n=7)	**P9-10 (n=8)**	**P17-25 (n=8)**	**P60-75 (n=35)**
Peak amplitude (pA)	-60 ± 7	**-36 ± 2	**-37 ± 2	-59 ± 2
Rise Time (ms)	**0.6 ± 0.1	**0.7 ± 0.1	**0.5 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	**-675 ± 62	**-331± 21	-181 ± 12	-242 ± 9
T70 (ms)	****14.6 ± 1**	****10.2 ± 0.4**	**5.8 ± 0.4**	**5.2 ± 0.2**
Tau w (ms)	****12.7 ± 1**	****10 ± 0.7**	****5.5 ± 0.3**	**4.0 ± 0.1**
Frequency (Hz)	**1.7 ± 0.2	**3.3 ± 0.5	**8.7 ± 1.5	17 ± 2

**$P < 0.05$; One-way ANOVA, in comparison to P60-75

The mIPSC decay (τ_W) of cortical layer 2/3 neurones decreased significantly with development: (P6-7 = 12.7 ± 1.2 ms, n = 7; P9-10 = 10 ± 0.7 ms, n = 8; P17-25 = 5.5 ± 0.3 ms, n = 8; P60-75 = 4 ± 0.1 ms, n = 35; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between all groups, $P < 0.05$; Table 5; Figure 9). As expected, in common with the τ_W, the mIPSC T_{70} also decreased significantly with development (P6-7= 14.6 ± 1.2 ms, n = 7; P9-10 = 10.2 ± 0.4 ms, n = 8; P17-25 = 5.8 ± 0.4 ms, n = 8; P60-75 = 5.2 ± 0.2 ms, n = 35). All the intergroup differences were statistically significantly different from each other except P17-25 *vs.* P60-75 (One-way ANOVA, with *post hoc* Newman Keul's test $P < 0.01$; Table 5). These data are consistent with previous work from the host laboratory, but also extend the observed changes to adult mice (P60-75).

3.5 The acute application of allopregnanolone prolongs the decay time of $GABA_AR$ mIPSCs more effectively in spinal LII neurones from older mice.

The neurosteroid allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and a closely related synthetic structural analogue ganaxolone (3β-methyl-3α-ol-5α-pregnan-20-one) have proven efficacy in enhancing the function of synaptic $GABA_ARs$ in other regions of the central nervous system such as the dentate gyrus and the rat spinal cord (Belelli & Herd 2003; Mitchell *et al.,* 2007). Allopregnanolone (1μM) was bath-applied following at least four minutes of stable control recording. Previous work by a former laboratory colleague has revealed an endogenous neurosteroid tone in the cortical neurones of immature mice (P8-11), sufficient to influence the time course of mIPSCs (Brown, 2012). Work at other centres has also suggested that a similar phenomenon may be observed in the LII neurones of the dorsal horn of the spinal cord (Poisbeau *et al*., 2005, Rajalu *et al*., 2009). I investigated the influence of the neurosteroid on the mIPSCs of LII neurones at two developmental stages: P8-11 and P17-25. Particular attention was given to the putative actions of the steroid to prolong the mIPSC decay time, as previous studies have revealed this to be a sensitive parameter (Mitchell *et al.,* 2007). For P8-11 neurones the mean τ_W was increased by 22 ± 6 % (n = 7) by 1μM allopregnanolone. By contrast, for P17-25 neurones the steroid appeared more

effective, increasing τ_W by 97 ± 47% (P8-11, control = 20 ± 1.7ms, 1μM allopregnanolone = 24.5 ± 2.4ms, n = 7, Student's paired t test, *P* <0.05; P17-25, control = 21.5 ± 5ms, allopregnanolone = 39.8 ± 9ms, n = 6, Student's paired t test, *P* <0.05). When the effect of 1μM allopregnanolone was normalised for comparison, the neurosteroid had a greater impact on $GABA_AR$ mIPSC τ_W at the later stage of development (Two-way RM ANOVA, *P* <0.05; Table 6; Figure 12). As described above, the parameter of T_{70} can readily be determined for each mIPSC and the impact of the steroid on this parameter differed for the two age groups. At P8-11 the T_{70} was increased by 34 ± 8% (n = 7) by 1μM allopregnanolone, whereas again the steroid appeared more effective on P17-25 neurons, the mean T_{70} increased by 68 ± 29%, n = 6 (P8-11, control = 20.2 ± 1.7ms, allopregnanolone = 26.5 ± 2.3ms, Student's paired t test, *P* <0.05; P17-25, control = 23.6 ± 6ms, allopregnanolone = 39 ± 10ms, Student's paired t test, *P* <0.05). When the effect of 1μM allopregnanolone was normalised for comparison, the neurosteroid had a greater impact on $GABA_AR$ mIPSC T_{70} at the later stage of development, Two-way RM ANOVA, *P* <0.05; Table 6; Figure 12). Studies from the host laboratory have previously reported a similar observation for LII neurones of rats (P15 - P21; Mitchell *et al.,* 2007). These data are consistent with the hypothesis that the neurosteroid tone of LII neurones decreases with development. However, other factors such as changes in the relative proportion of different types of subunit and altered neurosteroid-sensitivity of the synaptic $GABA_ARs$ may also be of significance (these issues are explored in the Discussion).

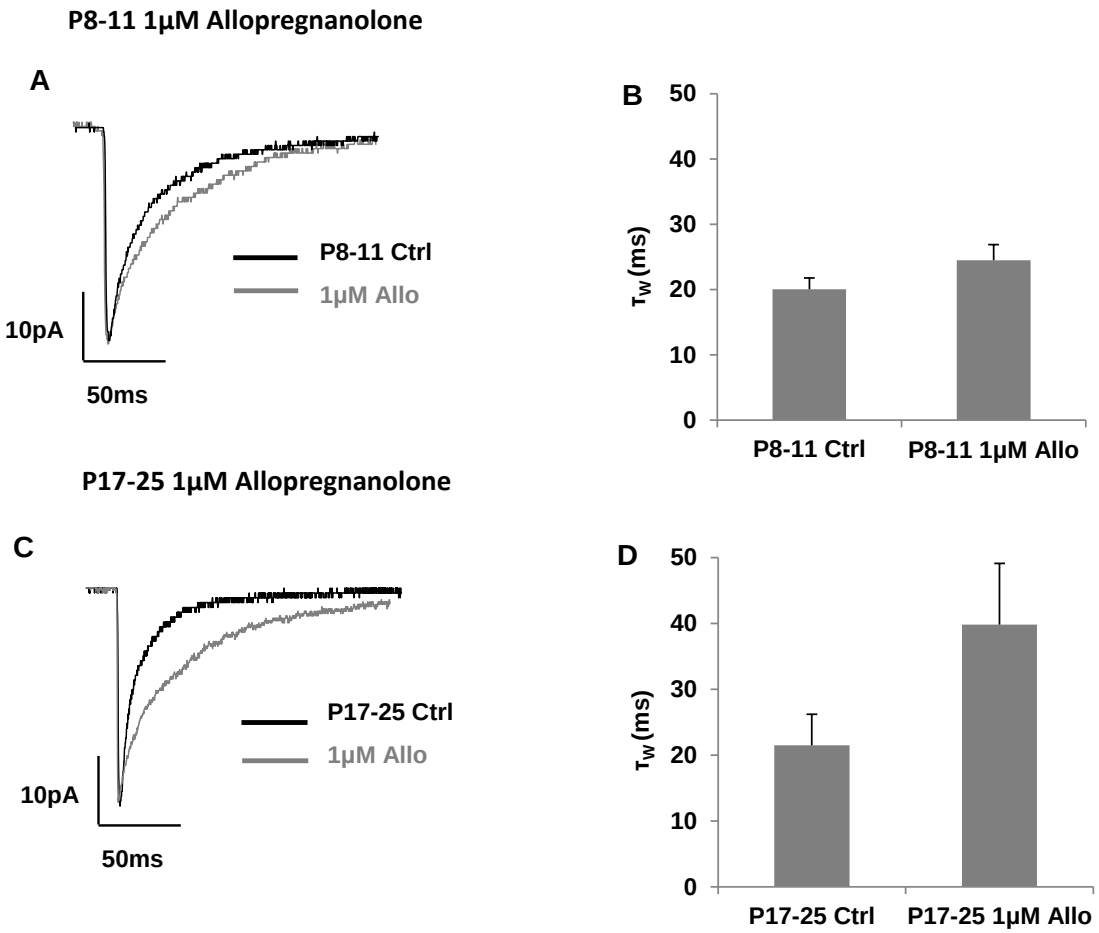

Figure 12 Acute exposure (10-20mins) to allopregnanolone (1μM) has a greater effect on the function of synaptic $GABA_A$Rs from mature spinal LII neurones compared to immature neurones.

(A & **C)** Superimposed exemplar $GABA_A$R-mediated mIPSCs from LII spinal neurones, aged P8-11 and P17-25 respectively, before and after acute exposure to 1μM allopregnanolone. **(B** & **D**) Histograms illustrating the relative magnitude of effect of allopregnanolone at the two stages of development. For P8-11 neurones the mean τ_w was increased by 22 ± 6 % (n = 7) by 1μM allopregnanolone. By contrast, for P17-25 neurones, the steroid appeared more effective, increasing τ_w by 97 ± 47% (P8-11, control = 20 ± 1.7ms, 1μM allopregnanolone = 24.5 ± 2.4ms, n = 7; P17-25, control = 21.5 ± 5ms, allopregnanolone = 39.8 ± 9ms, n = 6; Two-way RM ANOVA P <0.05). Allo = allopregnanolone, Ctrl = control.

Table 6 The acute application of allopregnanolone (Allo) prolongs the decay time of $GABA_AR$ mIPSCs more effectively in spinal LII neurones from older mice.

	P8-11 Control (n=7)	**P8-11 1µM Allo (n=7)**	**P17-25 Control (n=6)**	**P17-25 1µM Allo (n=6)**
Peak amplitude (pA)	-39 ± 5	-51 ± 7	-39 ± 7	-45 ± 5
Rise Time (ms)	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Charge transfer (fC)	-703 ± 81	*-1154 ± 207	-907 ± 296	-1566 ± 396
T70 (ms)	**20.2 ± 1.7**	***26.5 ± 2.3**	**23.6 ± 6**	***39.0 ± 10**
Tau w (ms)	**20 ± 1.7**	***24.5 ± 2.4**	**21.5 ± 5**	***39.8 ± 9**
Frequency (Hz)	2 ± 0.9	1 ± 0.3	0.5 ± 0.1	0.4 ± 0.1

*P < 0.05; paired Student's t test, in comparison to control

3.6 The acute application of ganaxolone prolongs the decay time of $GABA_AR$ mIPSCs in nRT neurones (P17-25).

Ganaxolone 1µM was bath-applied after obtaining at least four minutes of stable control recording and induced a prolongation of the exponential decay time of mIPSCs (τ_W: control = 15.2 ± 1.2 ms, n = 6; ganaxolone 1µM = 18.2 ± 1.8 ms, n = 6; Student's paired t test, P <0.05; Table 7; Figure 13). Similarly, the T_{70} was prolonged by steroid treatment: (control = 18.1 ± 1.2 ms, n = 6; ganaxolone 1µM = 21.7 ± 2 ms, n = 6; Student's paired t test, P <0.05; Table 7). These data confirm the activity of neurosteroids in the nRT.

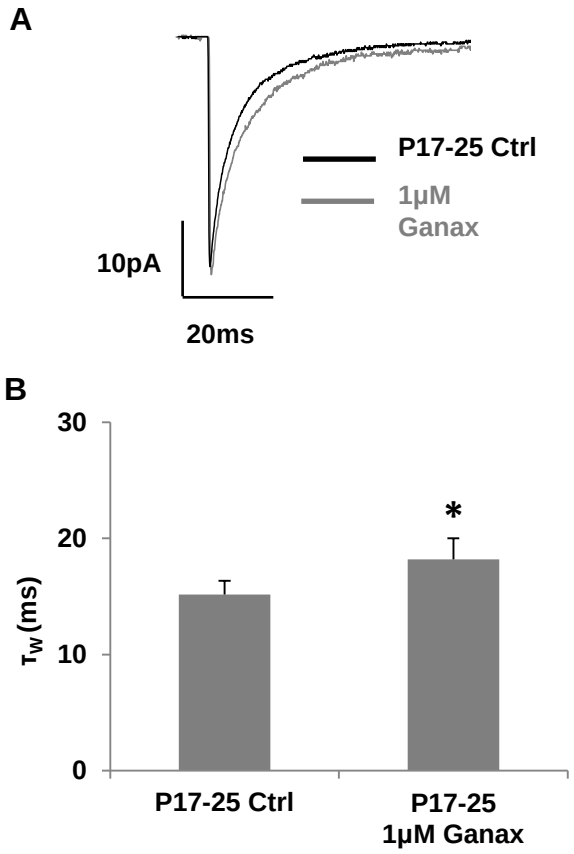

Figure 13 Acute exposure (10-20mins) of nRT neurones to ganaxolone (1μM) modestly enhances the function of synaptic $GABA_A$Rs.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from nRT neurones aged P17-25 before and after acute exposure to 1μM ganaxolone. **(B)** Histogram illustrating the significant effect of 1μM ganaxolone on $GABA_A$R mIPSCs of nRT neurones (τ_w in ms: P17-25 control = 15.2 ± 1.2ms, n = 6; P17-25 1μM ganaxolone = 18.2 ± 1.8ms; Student's paired t test *P <0.05; n = 6). Ganax = ganaxolone, Ctrl = control.

Table 7 The acute application of ganaxolone (Ganax) prolongs the decay time of $GABA_AR$ mIPSCs in nRT neurones (P17-25).

	P17-25 nRT Control (n=6)	**P17-25 nRT 1µM Ganax (n=6)**
Peak amplitude (pA)	-49 ± 4	-49 ± 4
Rise Time (ms)	0.6 ± 0.1	0.6 ± 0.1
Charge transfer (fC)	-721 ± 94	-807 ± 113
T70 (ms)	**18.1 ± 1.2**	***21.7 ± 2**
Tau w (ms)	**15.2 ± 1.2**	***18.2 ± 1.8**
Frequency (Hz)	6 ± 2	6 ± 3

*$P < 0.05$; paired Student's t test, in comparison to control

Table 8 The acute application of allopregnanolone (Allo) and its synthetic analogue ganaxolone (Ganax) prolongs the decay time of GABAAR mIPSCs of layer 2/3 cortical neurones from mature WT mice (P60-75).

	P60-75 Control (n=6)	**P60-75 1µM Allo (n=6)**	**P60-75 Control (n=6)**	**P60-75 1µM Ganax (n=6)**
Peak amplitude (pA)	-68 ± 3	-67 ± 3	-57 ± 4	*-66 ± 6
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-284 ± 7	-305 ± 14	-232 ± 15	*-309 ± 31
T70 (ms)	**5.1 ± 0.5**	***5.6 ± 0.5**	**5.2 ± 0.2**	***6.0 ± 0.4**
Tau w (ms)	**3.9 ± 0.3**	***4.4 ± 0.4**	**4.0 ± 0.2**	***4.7 ± 0.2**
Frequency (Hz)	29 ± 6	24 ± 3	22 ± 5	21 ± 5

*$P < 0.05$; paired Student's t test, in comparison to control

3.7 The acute application of allopregnanolone and its synthetic analogue ganaxolone prolongs the decay time of $GABA_AR$ mIPSCs of layer 2/3 neurones in mature WT mice (P60-75).

Allopregnanolone 1μM was bath-applied following at least four minutes of stable control recording. The steroid induced a modest prolongation of exponential decay time of $GABA_AR$ mIPSCs of mature layer 2/3 neurones (τ_W: control = 3.9 ± 0.3 ms, n = 6; allopregnanolone 1μM = 4.4 ± 0.4 ms, n = 6; Paired Student's t test, P <0.05; Table 8; Figure 14). Similarly, the mIPSC T_{70} was modestly prolonged by the steroid: (control = 5.1 ± 0.5 ms, n = 6; allopregnanolone 1μM = 5.6 ± 0.5 ms, n = 6; Student's paired t test, P <0.05; Table 8). Ganaxolone 1μM was introduced in a similar fashion and it also induced a modest prolongation of the mIPSC exponential decay time (τ_W: control = 4 ± 0.2 ms, n = 6; ganaxolone 1μM = 4.7 ± 0.2 ms, n = 6; Student's paired t test, P <0.05). Similarly, the mIPSC T_{70} was modestly prolonged by ganaxolone: (control = 5.2 ± 0.2 ms, ganaxolone 1μM = 6 ± 0.4 ms, n = 6; Student's paired t test, P <0.05; Table 8). When the data for τ_W and T_{70} were normalised for comparison, there was no significant difference between allopregnanolone (12 ± 3%) and ganaxolone (19 ± 4%) at P60-75 (Two-way RM ANOVA, P >0.05).

The acute application of a 3-fold greater concentration of ganaxolone (3μM) produced a similar prolongation of the mIPSC exponential decay time (20 ± 7%) to that caused by 1μM of this steroid (τ_W: control = 3.5 ± 0.2ms, n = 4; ganaxolone 3μM = 4.2 ± 0.2ms, n = 4; Student's paired t test, P <0.05; Figure 14). The mIPSC T_{70} was similarly prolonged by 3 μM ganaxolone (control = 4.8 ± 0.3ms, n = 4; ganaxolone 3μM = 5.9 ± 0.3ms, n = 4; Student's paired t test, P <0.05). When the data for τ_W and T_{70} were normalised for comparison, there was no significant difference between ganaxolone 1μM (12 ± 3%) and ganaxolone 3μM (20 ± 7%) at P60-75 (Two-way RM ANOVA, P >0.05).

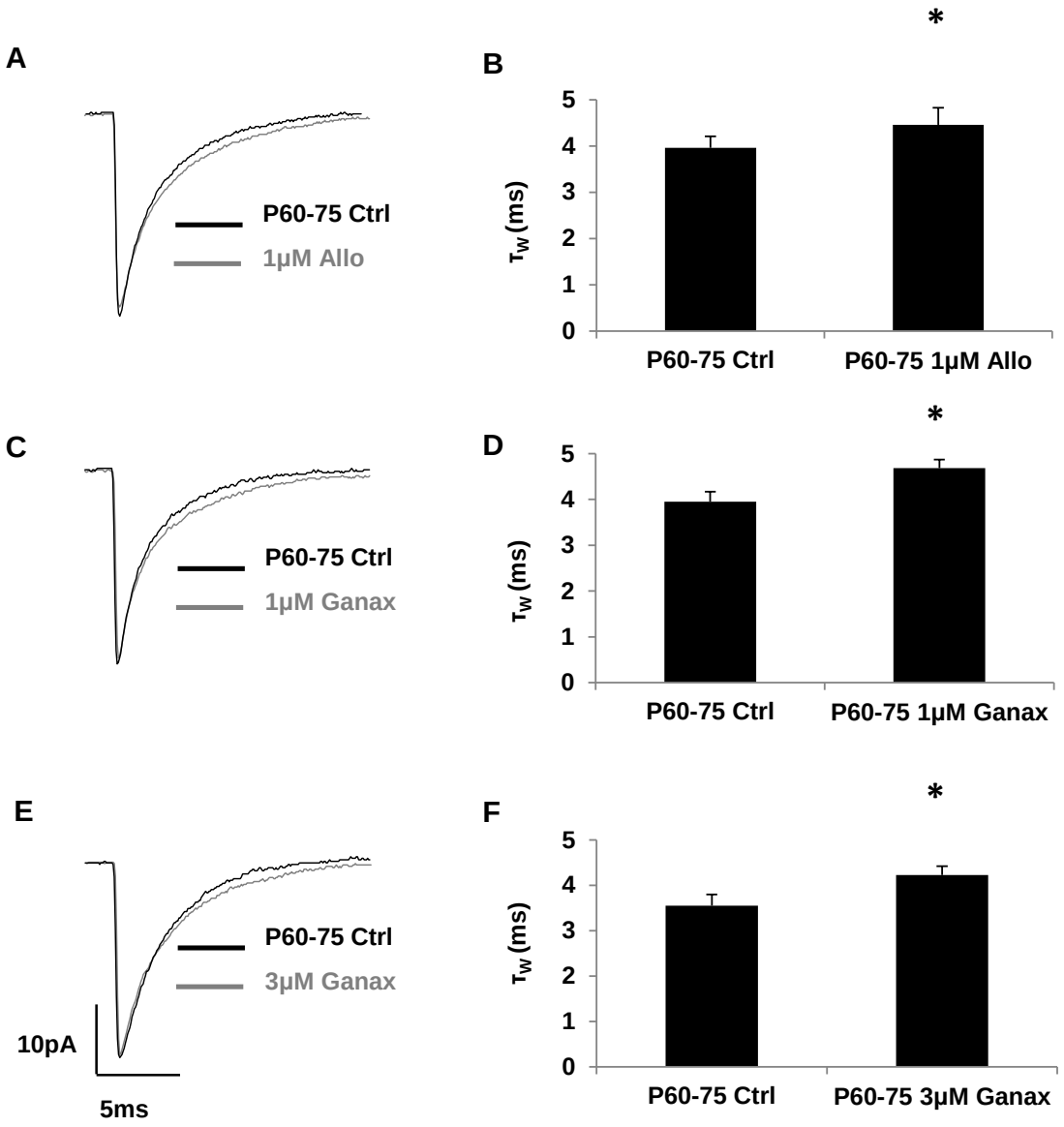

Figure 14 The acute exposure (10-20mins) of mature cortical neurones to allopregnanolone (1 μM), or ganaxolone (1-3 μM) modestly enhances the function of synaptic $GABA_A$Rs.

(A, C & E) Superimposed traces of exemplar $GABA_A$R-mediated mIPSCs from L2/3 cortical neurones aged P60-75 before and after acute exposure to 1μM allopregnanolone or 1-3 μM ganaxolone respectively. To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(B, D & F)** Histograms illustrating the significant effect of 1μM allopregnanolone and 1-3 μM ganaxolone on the time course of decay (τ_w in ms) of $GABA_A$R mIPSCs of L2/3 cortical neurones (Student's paired t test $^*P < 0.05$ for each drug treatment *vs.* its' paired control recording; n = 7).

3.8 The effect of γ-cyclodextrin (γ-CD) applied by three different methods on nRT $GABA_AR$ mIPSCs (P6-7).

Recordings were made from P6-7 nRT neurones using three methods of application of γ-CD to confirm which method was optimal. The γ-CD was presented within the recording electrode (intracellularly), applied *via* the extracellular solution, or by inclusion in the pipette, in the extracellular recording solution and in the incubation chamber containing the brain slice preparation prior to recording (*i.e.* 2 hours of preincubation). When γ-CD (1mM) was present in the extracellular solution (ECS) for at least 5 minutes, the mIPSC decay (τ_W) was not decreased significantly (P6-7 control = 31.1 ± 2 ms, n = 6, P6-7 γ -CD (ECS) = 28.7 ± 2.8 ms, n = 6; Student's paired t test, P <0.05; Table 9). Similarly, γ-CD had no significant effect on the mIPSC T_{70} (P6-7 control = 34.6 ± 2 ms, n = 6, P6-7 γ-CD (ECS) = 30.0 ± 2 ms, n = 6; Student's paired t test, P > 0.05; Table 9). When the γ-CD was present both intracellularly (0.5mM), extracellularly (1mM) and had previously been incubated with the brain slice preparation in the holding chamber (1mM) *i.e.* the "triple treatment", the mIPSC decay (τ_W) was significantly decreased. However, this protocol was no more effective than when the γ-CD was present only intracellularly (P6-7 control = 33.2 ± 1 ms, n = 24, P6-7 γ-CD ["triple treatment"] = 23.7 ± 1 ms, n = 13; Student's unpaired t test, P < 0.05; Table 10; Figure 15). The T_{70} was similarly decreased by this treatment regimen (P6-7 control = 35.8 ± 1 ms, n = 24; P6-7 γ-CD [triple treatment] = 22.3 ± 1 ms, n = 13; Student's unpaired t test, P < 0.01). These results indicate that intracellular application of γ-CD *via* the recording pipette is the optimal method of application and is consistent with the hypothesis that the $GABA_AR$-active neurosteroids are synthesised within the pain pathway neurones themselves (Akk *et al.,* 2009; Chisari *et al.,* 2009; Tsutsui, 2008). The effects of other types of cyclodextrin are explored in Results section 4.4.

Table 9 The effect of bath applied γ-cyclodextrin (CD) on nRT $GABA_AR$ mIPSCs (P6-7).

	P6-7 Paired Control (n= 6)	**P6-7 γ-CD in ECS only (n=6)**
Peak amplitude (pA)	-66 ± 6	-65 ± 4
Rise Time (ms)	0.7 ± 0.1	0.6 ± 0.1
Charge transfer (fC)	-1704 ± 208	-1557 ± 137
T70 (ms)	**34.6 ± 2**	***30.0 ± 2**
Tau w (ms)	**31.1 ± 2**	***28.7 ± 3**
Frequency (Hz)	3 ± 1	3 ± 1

*$P < 0.05$; paired Student's t test, in comparison to control

Table 10 The effect of γ-cyclodextrin (CD) applied by three different methods on nRT $GABA_AR$ mIPSCs (P6-7).

	P6-7 Control (n= 24)	**P6-7 γ-CD in ECS only (n=6)**	**P6-7 γ-CD in pipette only (n=8)**	**P6-7 γ-CD in pipette, ECS & storage chamber (n= 13)**
Peak amplitude (pA)	-56 ± 3	-65 ± 4	-52 ± 3	-44 ± 3
Rise Time (ms)	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Charge transfer (fC)	-1475 ± 83	-1557 ± 137	**-981 ± 81	**-946 ± 91
T70 (ms)	**35.8 ± 1**	**30.0 ± 2**	****22.0 ± 1**	****25.3 ± 1**
Tau w (ms)	**33.2 ± 1**	**28.7 ± 3**	****21.6 ± 1**	****23.7 ± 1**
Frequency (Hz)	2 ± 0.4	3 ± 1	3 ± 0.5	3.8 ± 0.7

**$P < 0.05$; One-way ANOVA, in comparison to P6-7 control

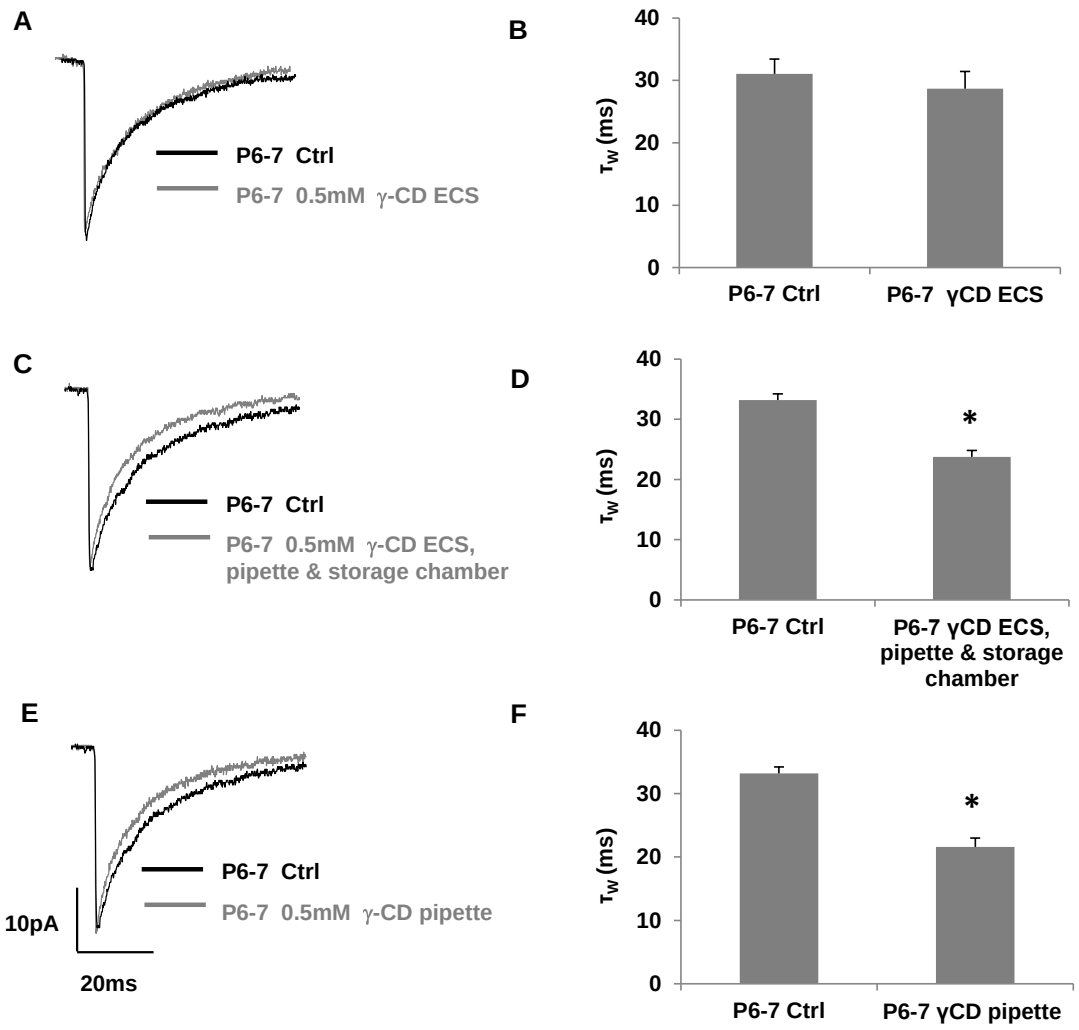

Figure 15 The effect of γ-CD on P6-7 nRT neurones varies according to the method of administration.

(A) Superimposed exemplar $GABA_A$Rs mIPSCs from nRT neurones aged P6-7 before and after acute exposure to 0.5mM γ-CD applied *via* the extracellular solution (ECS). **(B)** Histogram illustrating the lack of significant effect of extracellularly-applied 0.5mM γ-CD on $GABA_A$R mIPSCs of nRT neurones (P6-7 Ctrl = 31.1 ± 2 ms, n = 6, P6-7 γ -CD (ECS) = 28.7 ± 2.8 ms; Student's paired t test P >0.05; n = 6). **(C)** Superimposed exemplar $GABA_A$Rs mIPSCs from nRT neurones aged P6-7 illustrating the difference between unmatched control recordings and recordings made with 0.5mM γ-CD applied *via* the ECS, within the pipette and within the incubating chamber prior to recording. **(D)** Histogram illustrating the significant effect of 0.5mM γ-CD applied *via* the three methods described above in comparison to unmatched control recordings (P6-7 Ctrl = 33.2 ± 1 ms, n = 24; P6-7 γ-CD ["triple treatment"] = 23.7 ± 1ms, n = 13; Student's unpaired t test P < 0.05). **(E)** Superimposed exemplar $GABA_A$Rs mIPSCs from nRT neurones aged P6-7 illustrating the difference between unmatched control recordings and recordings made with 0.5mM γ-CD applied only intracellularly *via* the recording pipette. **(F)** Histogram illustrating the significant effect of 0.5mM γ-CD applied only intracellularly *via* the recording pipette on $GABA_A$R mIPSCs of nRT neurones aged P6-7 (P6-7 Ctrl = 33.2 ± 1 ms, n = 24; P6-7 γ-CD pipette = 21.6 ± 1ms, n = 8; Student's unpaired t test P < 0.05 in comparison to unmatched control recordings). These results indicate that intracellular application of γ-CD *via* the recording pipette is the optimal method of application and is consistent with the hypothesis that the $GABA_A$R-active neurosteroids are synthesised within the pain pathway neurones themselves. CD = cyclodextrin; ECS = extracellular solution; Ctrl = control.

3.9 The effect of γ-CD 0.5mM in the recording pipette on nRT $GABA_AR$-mediated mIPSCs at three stages of development.

γ-CD is a barrel shaped molecule that has been shown to sequester endogenous neurosteroid compounds within cortical neurones (Brown, 2012). Thus, this large hydrophilic molecule can be used as an experimental tool to reverse the modulatory action of endogenous neurosteroid (Shu *et al.,* 2007). Recordings were made at three stages of development in order to explore fluctuations in the endogenous neurosteroid tone of nRT neurones from P6–P25. γ-CD 0.5mM was present only within the recording electrode. The rationale for this method of application will be discussed subsequently.

Note in this study recordings were made and compared from a population of control and γ-CD treated (in the recording pipette) nRT neurones *i.e.* the recordings were not paired. The mIPSC decay (τ_W) of P6-7 nRT neurones was decreased by the presence of γ-CD (P6-7 control = 33.2 ± 1 ms, n = 24, P6-7 γ-CD = 21.6 ± 1.4 ms, n = 8; Student's unpaired t test, P <0.01 Table 11; Figure 16). The T_{70} was similarly decreased by γ-CD (P6-7 control = 36 ± 0.4 ms, n = 24, P6-7 γ-CD = 22.2 ± 0.2 ms, n = 24; Student's unpaired t test, P <0.01; Table 11). Consistent with these observations, the parameter of mIPSC charge transfer (*i.e.* the mIPSC area) was also decreased by γ-CD (Table 11).

The mIPSC decay (τ_W) of P10 nRT neurones was not decreased significantly by the presence of γ-CD (P10 control = 22.5 ± 0.7 ms, n = 14, P10 γ-CD = 22.1 ± 0.6 ms, n = 13; Student's unpaired t test, < 0.05; Table 11; Figure 16). The T_{70} was similarly unchanged by γ-CD (P10 control 25.4 ± 0.8 ms, n = 14, P10 γ-CD = 25 ± 0.6 ms, n = 13; Student's unpaired t test, >0.05). The parameter of mIPSC charge transfer (*i.e.* the mIPSC area) was also unchanged by γ-CD (Table 11).

The mIPSC decay (τ_W) of P17-25 nRT neurones was not decreased by the presence of γ-CD (P17-25 control = 18.2 ± 0.6 ms, n = 21, P17-25 γ-CD = 19 ± 1 ms, n = 15; Student's unpaired t test, >0.05; Table 11 Figure 16). Similarly, the T_{70} was not significantly decreased by γ-CD (P17-25 = 20.1 ± 0.6 ms, n = 21, P17-25 γ-CD = 19.3 ± 1 ms, n = 15; Student's unpaired t test, >0.05). The parameter of mIPSC charge transfer (*ie.* the mIPSC area) was unchanged by γ-CD (Table 11).

Table 11 The effect of γ-cyclodextrin (CD) 0.5mM in the recording pipette on nRT $GABA_A$R-mediated mIPSCs at three stages of development.

	P6-7 Control (n= 24)	P6-7 γ-CD (n=8)	P10 Control (n= 14)	P10 γ-CD (n= 13)	P17-24 Control (n=21)	P17-24 γ-CD (n=15)
Peak amplitude (pA)	-56 ± 3	-52 ± 3	-54 ± 2	-56 ± 3	-46 ± 1	-47 ± 3
Rise Time (ms)	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 1	0.6 ± 0.1	0.6 ± 1
Charge transfer (fC)	-1475 ± 83	*-981 ± 81	-1089 ± 51	-1131 ± 69	-745 ± 28	-771 ± 50
T70 (ms)	**35.8 ± 1**	***22.0 ± 1.4**	**25.4 ± 0.8**	**25.0 ± 0.6**	**20.1 ± 0.6**	**19.3 ± 1**
Tau w (ms)	**33.2 ± 1**	***21.6 ± 1.4**	**22.5 ± 0.7**	**22.1 ± 0.6**	**18.2 ± 0.6**	**19.0 ± 1**
Frequency (Hz)	2 ± 0.4	3 ± 0.5	7 ± 0.7	7 ± 0.8	8 ± 0.8	8 ± 1

*$P < 0.05$; unpaired Student's t test, in comparison to age-matched control

Table 12 Pipette-applied γ-cyclodextrin (CD) reduces decay time of $GABA_A$ mIPSCs of layer 2/3 cortical neurones at two stages of development (P9-10 and P60-75).

	P9-10 Control (n=8)	P9-10 γ-CD (n=6)	P60-75 Control (n=35)	P60-75 γ-CD (n=15)
Peak amplitude (pA)	-36 ± 2	-35 ± 3	-59 ± 2	-58 ± 2
Rise Time (ms)	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-333± 20	*-266 ± 22	-242 ± 9	*-185 ± 8
T70 (ms)	**10.3 ± 0.3**	***8.7 ± 0.6**	**5.2 ± 0.2**	***3.7 ± 0.2**
Tau w (ms)	**10.0 ± 0.6**	***7.4 ± 0.4**	**4.0 ± 0.1**	***2.9 ±0.1**
Frequency (Hz)	3 ± 0.5	*1.6 ± 0.5	17 ± 2	18 ± 2

*$P < 0.05$; unpaired Student's t test, in comparison to age-matched control

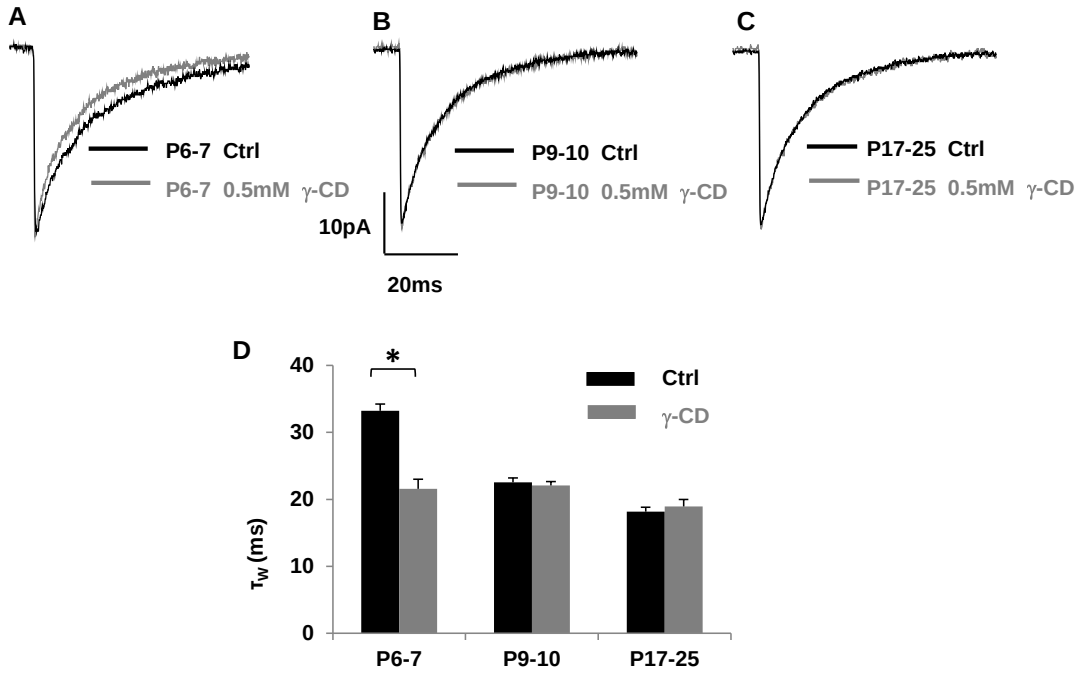

Figure 16 Effect of γ-CD applied intracellularly on nRT neurones varies depending on stage of development.

(A) Superimposed exemplar $GABA_A$Rs mIPSCs from nRT neurones aged P6-7 illustrating the difference between unmatched control recordings and recordings made with 0.5mM γ-CD applied only intracellularly *via* the recording pipette. **(B** & **C)** Superimposed exemplar $GABA_A$Rs mIPSCs from nRT neurones aged P9-10 and P17-25 respectively illustrating the lack of effect of 0.5mM γ-CD applied only intracellularly *via* the recording pipette at these stages of development. **(D)** Histogram illustrating the significant effect of 0.5mM γ-CD applied only intracellularly *via* the recording pipette on $GABA_A$R mIPSCs of nRT neurones aged P6-7 (P6-7 Ctrl = 33.2 ± 1 ms; P6-7 γ-CD pipette = 21.6 ± 1ms; $P < 0.05$) but not P9-10 (P9-10 Ctrl = 22.5 ± 0.7 ms; P9-10 γ-CD pipette = 22.1 ± 0.6ms; $P > 0.05$) or P17-25 (P17-25 Ctrl = 18.1 ± 0.6 ms; P17-25 γ-CD pipette = 19 ± 1ms; Student's unpaired t test $P > 0.05$ *vs* unmatched control; n = 8-24). CD = cyclodextrin; Ctrl = control.

3.10 Pipette-applied γ-CD reduces decay time of $GABA_AR$ mIPSCs of layer 2/3 cortical neurones at two stages of development.

To follow on from previous work in the nRT, recordings were made from L2/3 cortical neurones at two stages of maturity (P9-10 and P60-75) with γ-CD applied intracellularly. The mIPSC decay (τ_W) of P9-10 cortical neurones was significantly decreased in the presence of γ-CD (P9-10 control = 10.0 ± 0.6 ms, n = 8, P9-10 γ-CD = 7.4 ± 0.4 ms, n = 6; Student's unpaired t test, $P < 0.05$; Table 12; Figure 17). The T_{70} was similarly decreased by γ-CD treatment (P9-10 control = 10.3 ± 0.3 ms, n=8, P9-10 γ-CD = 8.7 ± 0.6 ms, n =6; Student's unpaired t test, $P <0.05$; Table 12).

The mIPSC decay (τ_W) of P60-75 cortical neurones was significantly decreased in the presence of γ-CD (P60-75 control = 4.0 ± 0.1 ms, n = 35, P 60-75 γ-CD = 2.9 ± 0.1 ms, n = 15; Student's unpaired t test, $P <0.05$; Table 12; Figure 17). The T_{70} was similarly decreased by γ-CD (P60-75 control = 5.2 ± 0.2 ms, n = 35, P60-75 γ-CD = 3.7 ± 0.2 ms, n = 15; Student's unpaired t test, $P <0.05$; Table 12). These data are in contrast to the lack of effect of γ-CD observed in nRT neurones at P9-10 and P17-24 (Figure 16). However, the data for P9/10 L2/3 cortical neurons are consistent with data obtained by a former laboratory colleague (Brown, 2012). Different regions of the nervous system reach maturation at different ages and it is possible that this may account for the regional variations observed in these experiments. Perhaps the most interesting finding is that the endogenous neurosteroid tone that previously appeared to be lost with maturation re-emerges in the adult mouse cortex, which may have a significant physiological role.

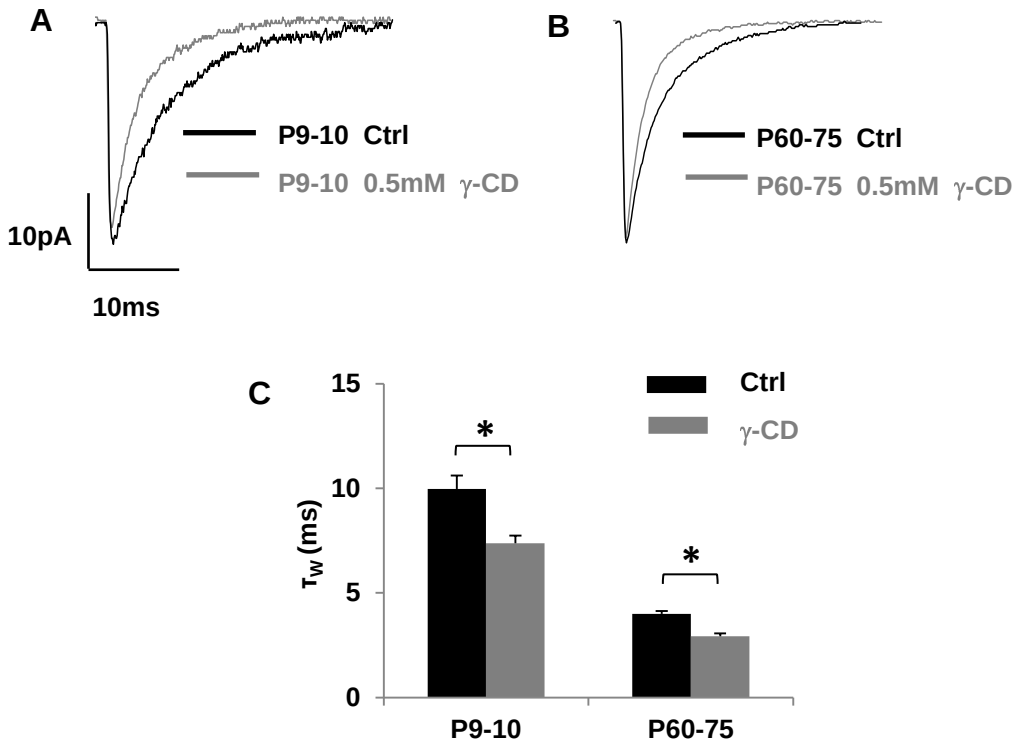

Figure 17 γ-CD applied intracellularly shortens the duration of $GABA_AR$ mIPSCs in L2/3 cortical neurones at age P9-10 & P60-75.

(A & **B)** Superimposed exemplar $GABA_ARs$ mIPSCs from L2/3 cortical neurones aged P9-10 and P60-75 respectively illustrating the significant effect of 0.5mM γ-CD applied only intracellularly *via* the recording pipette at these two stages of development. **(C)** Histogram illustrating the significant effect of 0.5mM γ-CD applied only intracellularly *via* the recording pipette on $GABA_AR$ mIPSCs of L2/3 cortical neurones aged P9-10 (P9-10 Ctrl = 10.0 ± 0.6 ms, n = 8, P9-10 γ-CD = 7.4 ± 0.4 ms, n = 6; $P < 0.05$) and P60-75 (P60-75 Ctrl = 4.0 ± 0.1ms, n = 35, P 60-75 γ-CD = 2.9 ± 0.1ms, n = 15; Student's unpaired t test $P < 0.05$ *vs* unmatched control). These results are consistent with the idea that there is an endogenous neurosteroid tone present in early development in pain pathway neurones, which decreases during maturation, but can reappear with full maturity. CD = cyclodextrin; Ctrl = control

Chapter 4: The role of neurosteroids on mature layer 2/3 neurones from the cerebral cortex of WT mice.

Neurosteroids are effective modulators of the $GABA_AR$ and have an important role in neurodevelopment and the maintenance of physiological inhibitory tone (Agis-Balboa *et al.,* 2006; Baulieu *et al.,* 2001; Belelli & Lambert, 2005; Belelli *et al.,* 2006). Changes in the levels of endogenous neurosteroids occur with certain forms of depression, epilepsy, schizophrenia and Alzheimer's disease and therefore manipulation of their levels could be of therapeutic benefit (Belelli *et al.,* 2006; Gunn *et al.,* 2011; Luchetti *et al.,* 2011; Reddy, 2010). Of particular relevance to the present study, neurosteroid synthesis in dorsal horn neurones of the spinal cord is up-regulated in response to inflammatory pain to provide a form of endogenous analgesia (Poisbeau *et al.,* 2005). As detailed in Part 3 of the Introduction, the synthesis pathway for allopregnanolone is as follows: 1) pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). 2) progesterone is converted to 5α-dihydroxyprogesterone (DHP) by 5α-reductase (5α-R). 3) DHP is converted to allopregnanolone (5α3α) by 3α-hydroxysteroid dehydrogenase (3α-HSD). In addition, the active compound allopregnanolone may be converted back to DHP by 3α-HSD, or to the inactive epiallopregnanolone (3β5α) (Belelli & Lambert, 2005; Mellon *et al.,* 2001; Rupprecht *et al.,* 2010; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003; Figure 3.)

The first results chapter described how endogenous neurosteroid tone fluctuates with development and the impact that this has on $GABA_AR$-mediated mIPSCs of neurones known to be part of a pain pathway. This chapter describes the influence of neurosteroids on cortical mIPSCs recorded from brain slice preparations made from adult (P60-75) WT mice. The steroid was administered i) acutely, ii) as a two-hour brain slice incubation treatment or iii) delivered to the intracellular compartment of the neurone *via* the recording pipette. Additionally, the influence of cyclodextrin on the endogenous neurosteroid tone was investigated. It is technically more challenging to obtain whole-cell voltage-clamp recordings from mice aged P60-75, than from younger animals. Typically only neurones from deeper within the slice are viable and the slice tissue is denser in older animals, which makes the visualisation of neurones more challenging. In Chapter 3, the properties of certain neurones of the pain pathway (spinal cord, thalamic and cortical neurones) were investigated at different stages of development. As the

behavioural studies were to be conducted in adult mice, it was decided to make recordings from adult layer 2/3 cortical neurones for three reasons: 1) viable recordings of nRT neurones of older animals are compromised by the high density of axonal projections (Cox *et al.,* 1997; Pinault & Deschenes, 1998). 2) values for the mean τ_W of L2/3 cortical $GABA_AR$ mIPSCs are relatively homogenous, in contrast to the mean τ_W values of $GABA_AR$ mIPSCs of LII neurones which are heterogenous, which makes inter-group comparison more difficult (as detailed in the previous chapter; Figure 11). 3) Layer 2/3 cortical neurones are part of the pain pathway.

4.1 The effect of the acute application of allopregnanolone or ganaxolone on $GABA_AR$ mIPSCs in mature WT mice (P60-75).

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is an effective modulator of synaptic $GABA_AR$ function in multiple regions of the nervous system, including the spinal cord, thalamus, cerebral cortex and the hippocampus (Belelli & Herd, 2003; Belelli & Lambert, 2005; Brown, 2012; Inquimbert *et al.,* 2007; Keller *et al.,* 2004; Mitchell *et al.,* 2007). The neuroactive steroid ganaxolone, is a potent positive allosteric modulator of the $GABA_AR$ (Carter *et al.,* 1997; Belelli & Herd 2003). Ganaxolone is a metabolically stable synthetic analogue of allopregnanolone, the molecular structure of which differs only by having an extra methyl group (adjacent to the hydroxyl group at the 3 position of the A-ring) that prevents oxidation to an inactive form (Carter *et al.,* 1997). The extra methyl group means that *in vivo* it may have a longer duration of action than allopregnanolone, a feature which may enhance its therapeutic potential for conditions such as epilepsy, or neuropathic pain (Carter *et al.,* 1997). In order to enhance the flow and clarity of the thesis and to allow this Chapter to stand alone, data from section 3.7 referring to the acute application of allopregnanolone and ganaxolone is intentionally reiterated in this section (4.1).

Table 13 The acute application of allopregnanolone (Allo) or ganaxolone (Ganax) prolongs the decay time of $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT mice.

	WT Pre 1μM Allo (n=7)	**WT Post 1μM Allo (n=7)**	**WT Pre 1μM Ganax (n=6)**	**WT Post 1μM Ganax (n=6)**	**WT Pre 3μM Ganax (n=4)**	**WT Post 3μM Ganax (n=4)**
Peak amplitude (pA)	-68 ± 3	-67 ± 5	-57 ± 4	*-66 ± 6	-65 ± 2	-67 ± 5
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-284 ± 7	-305 ± 14	-232 ± 15	*-309 ± 31	-245 ± 21	-295 ± 21
T70 (ms)	**5.2 ± 0.4**	***5.8 ± 0.5**	**5.2 ± 0.2**	***6.1 ± 0.4**	**4.8 ± 0.3**	***5.9 ± 0.3**
Tau w (ms)	**4.0 ± 0.3**	***4.5 ± 0.4**	**4.0 ± 0.2**	***4.7 ± 0.2**	**3.6 ± 0.3**	***4.2 ± 0.2**
Frequency (Hz)	26 ± 6	21 ± 4	22 ± 5	21 ± 5	28 ± 3	31 ± 4

*$P < 0.05$; paired Student's t test, in comparison to age-matched control

n = number of neurones

Table 14 The effect of prolonged incubation of allopregnanolone (Allo) or ganaxolone (Ganax) on $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT mice

	WT Control (n=35)	**WT 100nM Allo (n=9)**	**WT 300nM Allo (n=9)**	**WT 30nM Ganax (n=10)**	**WT 100nM Ganax (n=10)**	**WT 300nM Ganax (n=10)**	**WT 1μM Ganax (n=10)**	**WT 3μM Ganax (n=8)**
Peak amplitude (pA)	-59 ± 2	**-78 ± 2	**-87 ± 5	**-61 ± 3	**-66 ± 4	**-82 ± 6	**-97 ± 9	**-153 ± 6
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Charge transfer (fC)	-242 ± 9	**-932 ± 84	**-1489 ± 136	**-300 ± 26	**-440 ± 39	**-749 ± 88	**-1575 ± 195	**-4396 ± 268
T70 (ms)	**5.2 ± 0.2**	****18.6 ± 1**	****27 ± 1**	****6.3 ± 0.4**	****9.6 ± 0.6**	****13.4 ± 1**	****24.2 ± 1**	****44.2 ± 2**
Tau w (ms)	**4.0 ± 0.1**	****12.5 ± 1**	****19 ± 1**	****5.1 ± 0.3**	****7.2 ± 0.5**	****10.9 ± 1**	****18.8 ± 1**	****32.5 ± 1**
Frequency (Hz)	17 ± 2	15 ± 3	**11 ± 1	**11 ± 1	15 ± 3	20 ± 3	13.5 ± 2	**8 ± 1

***P* < 0.05; One-way ANOVA in comparison to age-matched control

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones before and after the bath application of allopregnanolone, or ganaxolone. The neurosteroid was bath-applied after at least four minutes of stable recording, (during which time control mIPSCs were obtained) enabling the comparison of paired recordings. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, charge transfer, decay kinetics and frequency as detailed in Table 13.

After ten minutes application, allopregnanolone induced only a modest (12 ± 3%) prolongation of the exponential decay time in the $GABA_AR$-mediated mIPSCs of WT L2/3 cortical neurones (τ_W: control = 4.0 ± 0.3 ms, n = 7; allopregnanolone 1 μM = 4.5 ± 0.4 ms, n = 7; paired Student's t test, P <0.05; Table 13; Figure 18). In agreement with the effects of the steroid on the decay constant (τ_W) of the averaged mIPSC, the mIPSC T_{70} was similarly modestly prolonged by allopregnanolone (Table 13). It is conceivable that the limited effect of acute allopregnanalone is in part a consequence of local metabolism occurring within the slice to a GABA-inactive product (Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003). Therefore, I investigated the acute effects of ganaxolone, the metabolically stable analogue of allopregnanolone. However, in common with allopregnanolone, ganaxolone (1μM) induced only a modest (19 ± 4%) prolongation of exponential decay time of the $GABA_AR$-mediated mIPSCs of WT layer 2/3 cortical neurones after ten minutes application (τ_W: control = 4 ± 0.2 ms, n = 6; ganaxolone 1 μM = 4.7 ± 0.2 ms, n = 6; Student's paired t test, P <0.05; Table 13; Figure 18). Similarly, the mIPSC T_{70} was modestly prolonged by ganaxolone (Table 13). Furthermore, the acute application of a 3-fold greater concentration of ganaxolone (3μM) only produced a similar (20 ± 7%) prolongation of the $GABA_AR$ mIPSC exponential decay time of WT L2/3 cortical neurones to that caused by 1μM of this steroid (τ_W: control = 3.6 ± 0.3 ms, n = 4; ganaxolone 3 μM = 4.2 ± 0.2 ms, n = 4; Student's paired t test, P <0.05; Table 13; Figure 18). The mIPSC T_{70} was similarly modestly prolonged by 3 μM ganaxolone (Table 13). These results illustrate that ganaxolone modulates synaptic $GABA_AR$ function in mature L2/3 cortical neurones of WT mice when applied acutely and further suggest that the limited effects of allopregnanolone are not a consequence of local metabolism.

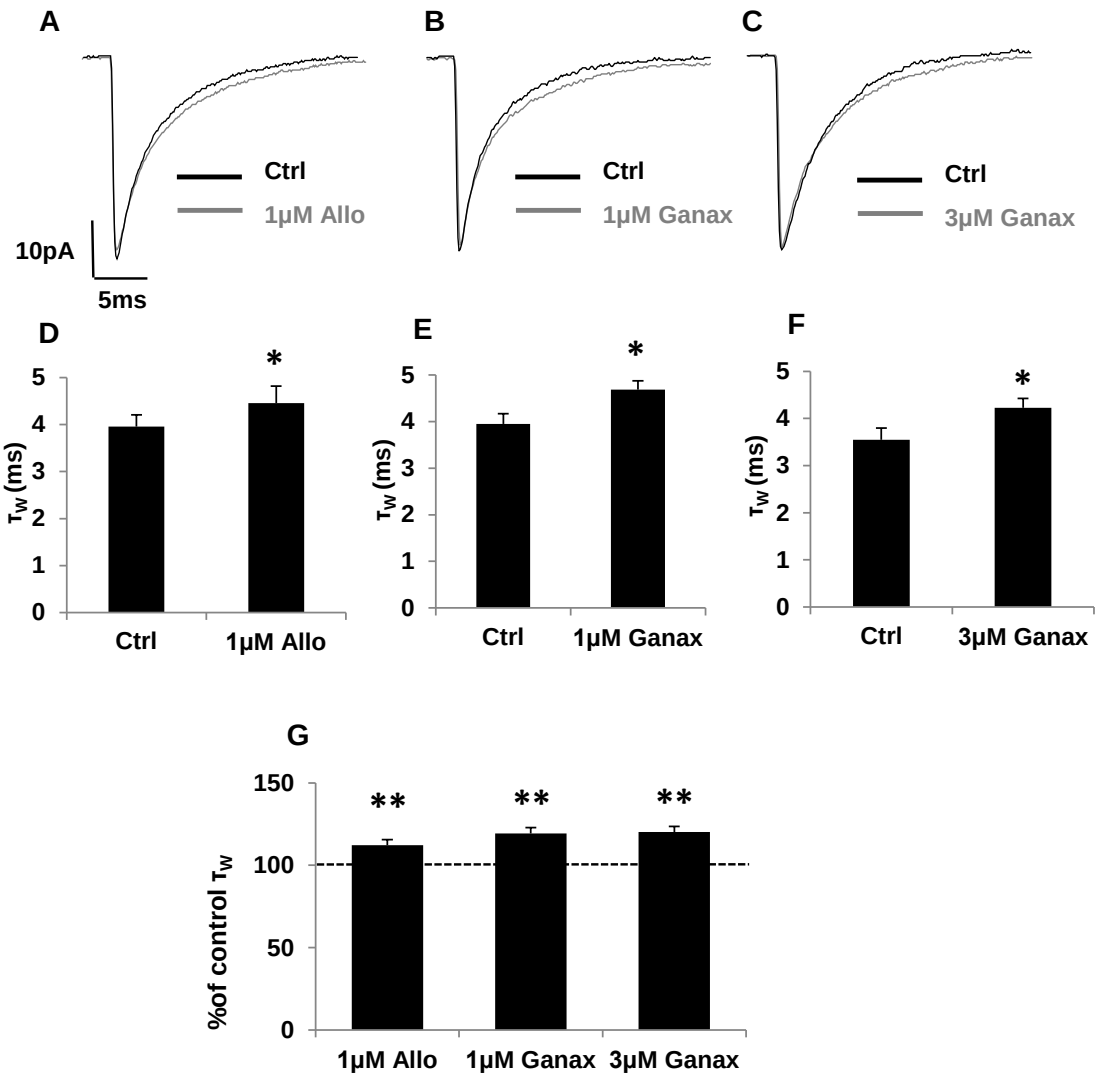

Figure 18 The acute exposure (10-20mins) of mature cortical neurones to allopregnanolone (1 μM), or ganaxolone (1-3 μM) modestly enhances the function of synaptic $GABA_AR$s.

(A-C) Superimposed traces of exemplar $GABA_AR$-mediated mIPSCs from L2/3 cortical neurones aged P60-75 before and after acute exposure to 1μM allopregnanolone or 1-3 μM ganaxolone respectively. To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(D-F)** Histograms illustrating the significant effect of 1 μM allopregnanolone and 1-3 μM ganaxolone on the time course of decay (τ_w in ms) of $GABA_AR$ mIPSCs of L2/3 cortical neurones (Student's paired t test *P <0.05 for each drug treatment *vs.* its' paired control recording; n = 7). **(G)** Histogram illustrating the effect of the neuroactive steroids with the data normalised relative to control recordings (*i.e.* expressed as a percentage of the control recording). When the data for τ_w was normalised to allow comparison of the effect of the two steroids, there was no significant difference between the effect produced by allopregnanolone 1 μM (12 ± 3%) and that produced by ganaxolone 1 μM (19 ± 4%), or by 3 μM (20 ± 7%) respectively (Two-way RM ANOVA **P >0.05; n = 4-7). Allo = allopregnanolone; Ganax = ganaxolone; Ctrl = control.

The relatively modest prolongation of $GABA_AR$ mIPSC decay time (even for the higher concentration of ganaxolone) raises the question of how well the neurosteroids are penetrating the brain slice. When the data for τ_W was normalised, there was no significant difference between allopregnanolone 1 μM (12 ± 3%), ganaxolone 1 μM (19 ± 4%) and 3μM (20 ± 7%) respectively (Two-way RM ANOVA, P >0.05). The next series of experiments explore the effect of incubating the preparation with the neurosteroids (allopregnanolone and ganaxolone) for at least two hours.

4.2 The effect of prolonged incubation of allopregnanolone (100 - 300 nM), or ganaxolone (30 nM - 3 μM), on cortical $GABA_AR$-mediated mIPSCs of adult WT mice.

The lipophilic intravenous anaesthetics etomidate and propofol, which in common with neuroactive steroids enhance the function of $GABA_ARs$, require relatively prolonged incubation times to approach equilibrium in a brain slice preparation (over 1-2 hours; Benkwitz *et al.,* 2007; Gredell *et al.,* 2004). Therefore in case the same is true for neursteroids, recordings were made after incubation treatment with allopregnanolone and ganaxolone.

In this set of experiments the analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 14). In contrast to the relatively modest prolongation of mIPSCs described above with an acute steroid application protocol, a two-hour incubation of the brain slice preparation with ~ 3 to 10 times lower concentrations of allopregnanolone (100 – 300 nM) produced a dramatic concentration-dependent increase of the WT layer 2/3 cortical $GABA_AR$ mIPSC exponential decay time (τ_W: control = 4.0 ± 0.1 ms, n = 35; allopregnanolone 100 nM = 12.5 ± 1 ms, n = 9; allopregnanolone 300 nM = 19.0 ± 1 ms, n = 9; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed significant differences between all three treatments and the two concentrations of allopregnanolone increased τ_W to 313 ± 25% and 480 ± 22% of control respectively, One-way RM ANOVA, P <0.05; Table 14; Figure 19). Similarly, the mIPSC T_{70} was significantly prolonged by allopregnanolone (Table 14).

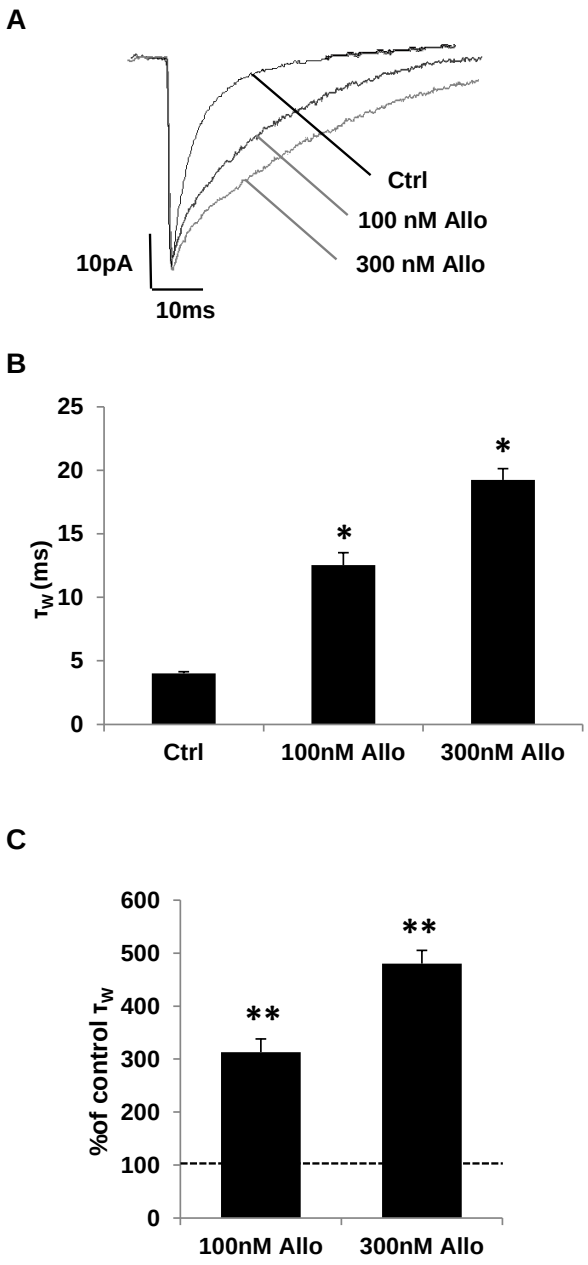

Figure 19 The prolonged exposure (~2 hrs) of mature cortical neurones to relatively low concentrations of allopregnanolone (100-300 nM) greatly enhances the function of synaptic $GABA_AR$s.

(A) Superimposed traces of exemplar $GABA_AR$-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after ~2 hour incubation of the brain slice with 100 nM and 300 nM allopregnanolone. To facilitate comparison of their time course the averaged mean mIPSCs are normalised to the peak amplitude of the representative control mean mIPSC. **(B)** Histogram illustrating the dramatic concentration-dependent effect of allopregnanolone on the duration of $GABA_AR$-mediated mIPSCs (the time course of decay- τ_w in ms; One-way ANOVA *P <0.05; n = 9-10). **(C)** Histogram illustrating the concentration-dependent effect of allopregnanolone on the duration of $GABA_AR$-mediated mIPSC (τ_w expressed as a percentage of control). Note 100 nM and 300 nM allopregnanolone increased τ_w to 313 ± 25% and 480 ± 22% of control respectively (One-way RM ANOVA P <0.05. *Post hoc* Newman Keul's test revealed significant differences between control and both concentrations of allopregnanolone **P <0.05 n = 9-10). Allo = allopregnanolone; Ctrl = control.

These results indicate that allopregnanolone is a potent modulator of synaptic $GABA_AR$s in mature WT L2/3 cortical neurones, but additionally demonstrate that the steroid effect is greatly underestimated when applied acutely. The large difference between acute bath application and the two-hour incubation is probably a consequence of the time required for the steroid to approach equilibrium within the brain slice (Benkwitz *et al.,* 2007; Gredell *et al.,* 2004). No such effect was observed in time matched controls. Of note, allopregnanolone may be converted back to DHP, or to the inactive epiallopregnanolone (3β5α) (Belelli & Lambert, 2005; Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003).

As described above, acute ganaxolone had a similar effect to allopregnanolone in causing only a modest prolongation of the $GABA_AR$-mediated mIPSC. A two-hour incubation of the brain slice preparation with ganaxolone produced a significant concentration-dependent increase of the WT L2/3 cortical $GABA_AR$ mIPSC exponential decay time, although the magnitude of the effect was less than that induced by allopregnanolone (τ_W: control = 4.0 ± 0.1 ms, n = 35; ganaxolone 30 nM = 5.1 ± 0.3 ms, n = 10; ganaxolone 100 nM = 7.2 ± 0.5 ms, n = 10; ganaxolone 300 nM = 10.9 ± 1 ms, n = 10; ganaxolone 1 μM = 18.8 ± 1 ms, n = 10; ganaxolone 3 μM = 32.5 ± 1 ms, n = 8; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed significant differences between all the concentrations of ganaxolone, τ_W was increased to 126 ± 8%, 180 ± 11%, 273 ± 32%, 468 ± 32% and 811 ± 42% of control respectively, One-way RM ANOVA, P <0.05; Table 14; Figure 20). Similarly, the mIPSC T_{70} was significantly prolonged by ganaxolone (Table 14). These results reveal that in common with allopregnanolone, the prolonged incubation of the brain tissue with ganaxolone is far more effective than the acute application protocol. However, although highly effective, ganaxolone appears less efficacious than allopregnanolone in modulating $GABA_AR$-mediated mIPSCs in mature layer 2/3 cortical neurones of WT mice. Indeed, allopregnanolone had greater potency than ganaxolone at the same concentrations: 100 nM (313 ± 25% *vs*. 180 ± 11%; One-way RM ANOVA, P <0.05) and 300 nM (480 ± 22% *vs*. 273 ± 32%; One-way RM ANOVA, P <0.05). (Table 14, Figure 21).

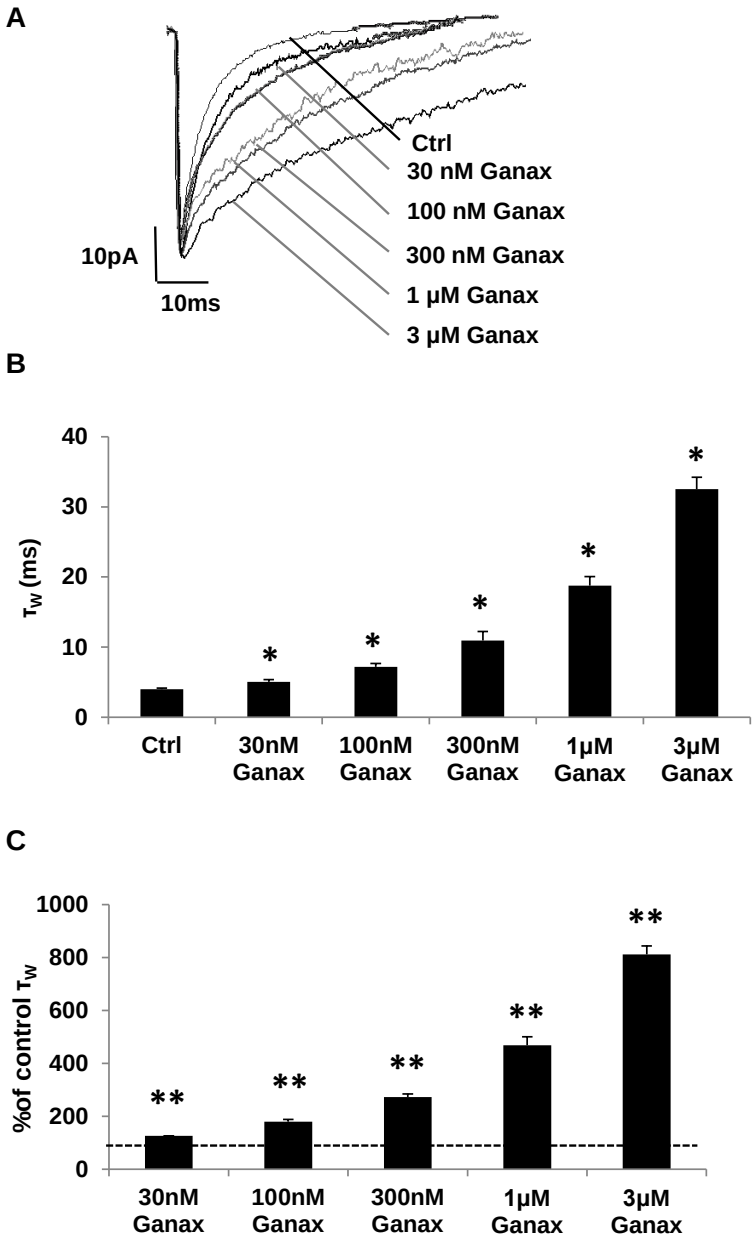

Figure 20 The prolonged exposure (~2 hrs) of mature cortical neurones to ganaxolone (30 nM- 3 μM) greatly enhances the function of synaptic $GABA_A$Rs in a concentration-dependent manner.

(A) Traces of superimposed exemplar $GABA_A$R-mediated mIPSCs from a representative control mature L2/3 cortical neurone and from equivalent neurones after ~2 hour incubation of the brain slice with 30 nM - 3 μM ganaxolone. **(B)** Histogram illustrating the dramatic concentration-dependent effect of ganaxolone on the duration of $GABA_A$R-mediated mIPSCs (the time course of decay, τ_w in ms; One-way ANOVA **P* <0.05; n = 8-10). **(C)** Histogram illustrating the concentration-dependent effect of ganaxolone on the duration of $GABA_A$R-mediated mIPSCs (τ_w expressed as a percentage of control). Note 30 nM - 3 μM ganaxolone increased τ_w to 126 ± 8%, 180 ± 11%, 273 ± 32%, 468 ± 32% and 811 ± 42% of control respectively (One-way RM ANOVA *P* <0.05. *Post hoc* Newman Keul's test revealed significant differences between all the concentrations of ganaxolone ***P* <0.05; n = 8-10). Ganax = ganaxolone; Ctrl = control.

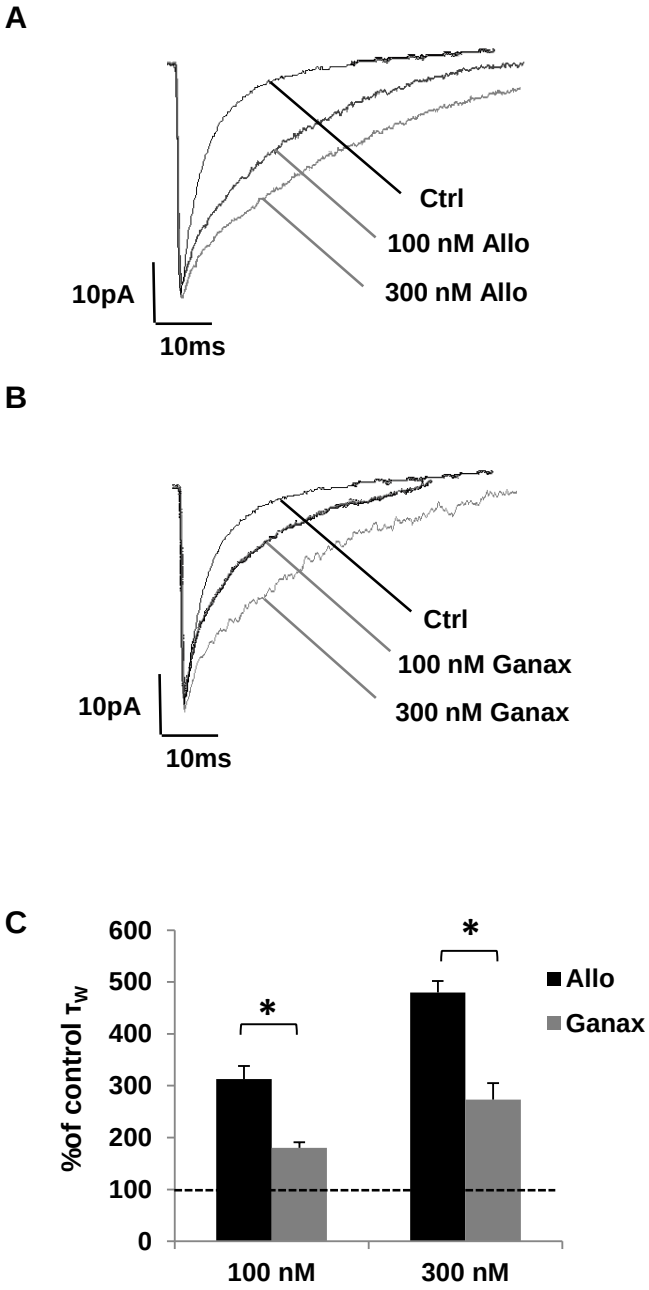

Figure 21 A comparison of the effects of prolonged exposure (~2 hrs) of mature cortical neurones to allopregnanolone (100-300 nM) and ganaxolone (100-300 nM) on synaptic $GABA_AR$ function.

(A & **B)** Superimposed exemplar $GABA_AR$-mediated mIPSCs acquired from representative control mature L2/3 cortical neurones and from equivalent neurones after an ~2 hour incubation of the brain slice with 100 nM and 300 nM allopregnanolone, or ganaxolone. **(C)** Histogram comparing the concentration-dependent effects of allopregnanolone and ganaxolone on the duration of $GABA_AR$-mediated mIPSCs (the time course of decay τ_w expressed as a percentage of control). The histogram illustrates the significantly greater effect of allopregnanolone compared to ganaxolone when tested at the same concentrations: 100 nM (313 ± 25% *vs*. 180 ± 11%; **P* <0.05; n = 9-10) and 300 nM (480 ± 22% *vs*. 273 ± 32%; One-way RM ANOVA ***P* <0.05; n = 9-10). Allo = allopregnanolone; Ganax = ganaxolone; Ctrl = control.

4.3 The effect of intracellular allopregnanolone or ganaxolone (3-10 μM) on layer 2/3 cortical $GABA_A$ mIPSCs in mature WT mice (P60-75)

As descried above, acute allopregnanolone had only a modest effect on the $GABA_AR$-mediated mIPSCs decay time, but was far more efficacious in this respect when brain tissue slices were incubated with steroid for > 2 hours, suggesting that the steroid is relatively slow to equilibrate within the tissue. It has been proposed that endogenous neurosteroids may be synthesised in the postsynaptic neurone and act in an autocrine manner to influence GABA-ergic transmission (Agis Balboa *et al.,* 2006; Lambert *et al.,* 2009). It is implicit in this model that intracellular steroid would modulate the $GABA_ARs$ of the postsynaptic neurone and the recording pipette can be employed to deliver drugs to the neurone interior (Evans & Marty, 1986). Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones with allopregnanolone, or ganaxolone present in the recording pipette to explore whether the steroid could modulate synaptic $GABA_ARs$ when delivered to the intracellular compartment. These recordings were compared to separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, charge transfer, decay kinetics and frequency as detailed in Table 15.

The presence of allopregnanolone in the recording pipette significantly increased the mIPSC decay of WT L2/3 cortical neurones in a concentration-dependent manner (τ_W: control = 4.0 ± 0.1 ms, n = 35; 3 μM allopregnanolone = 5.8 ± 0.8 ms, n = 6; 10 μM allopregnanolone = 16.1 ± 1.3 ms, n = 7; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between all three treatments, $P < 0.05$. Table 15, Figure 22). In agreement with the effects of the steroid on the decay constant (τ_W) of the averaged mIPSC, the mIPSC T_{70} was similarly modestly prolonged by allopregnanolone (Table 15). Here, the higher concentration of allopregnanolone (10 μM) had a comparatively large concentration-dependent effect on cortical $GABA_AR$ mIPSCs when presented acutely within the recording electrode. This finding indicates that neurosteroids are able to exert their effect *via* the intracellular compartment and is in agreement with the literature (Akk *et al.,* 2005; See Discussion section 7.4 for further details).

Table 15 The effect of intracellular allopregnanolone (Allo) or ganaxolone (Ganax) on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice

	WT Control (n=35)	WT 3μM Allo (n=6)	WT 10μM Allo (n=7)	WT 3μM Ganax (n=6)	WT 10μM Ganax (n=6)
Peak amp (pA)	-59 ± 2	-64 ± 3	**-95 ± 4	-63 ± 4	-69 ± 6
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	-354 ± 49	**-1389 ± 139	**-347 ± 15	**-420 ± 55
T70 (ms)	**5.2 ± 0.2**	**7.7 ± 1	**22.3 ± 1.4	**7.1 ± 0.4	**8.1 ± 0.6
Tau w (ms)	**4.0 ± 0.1**	**5.8 ± 0.8	**16.1 ± 1.3	**5.5 ± 0.4	**6.1 ± 0.3
Frequency (Hz)	17 ± 2	22 ± 4	20 ± 3	18 ± 4	16 ± 2

**$P < 0.05$; One-way ANOVA, in comparison to age-matched control

The concentrations of pipette-applied allopregnanolone are relatively high, but the time scale from application to recording is short (< 10 minutes) and dialysis rate of the intracellular contents may be an influential limiting factor. The presence of ganaxolone in the recording pipette also significantly increased the mIPSC decay of WT L2/3 cortical neurones in a concentration-dependent manner (τ_W: control = 4.0 ± 0.1 ms, n = 35; 3 μM ganaxolone = 5.5 ± 0.4 ms, n = 6; 10 μM ganaxolone = 6.1 ± 0.3 ms, n = 6; One-way ANOVA, $P < 0.05$; Table 15, Figure 23). In agreement with the effects of the steroid on the decay constant (τ_W) of the averaged mIPSC, the mIPSC T_{70} was similarly modestly prolonged by ganaxolone (Table 15). The effect of pipette-applied ganaxolone is consistent with the recordings described above with allopregnanolone, although ganaxolone had a less pronounced effect on $GABA_AR$ mIPSCs. Collectively these experiments illustrate that steroid penetration of brain slice tissue is a significant limiting factor, the intracellular application is an effective method of presenting neuroactive steroids to $GABA_ARs$ (Figure 24) and that, in these neurones, allopregnanolone is a more effective modulator of the synaptic $GABA_AR$ than ganaxolone (Figure 21 & Figure 25).

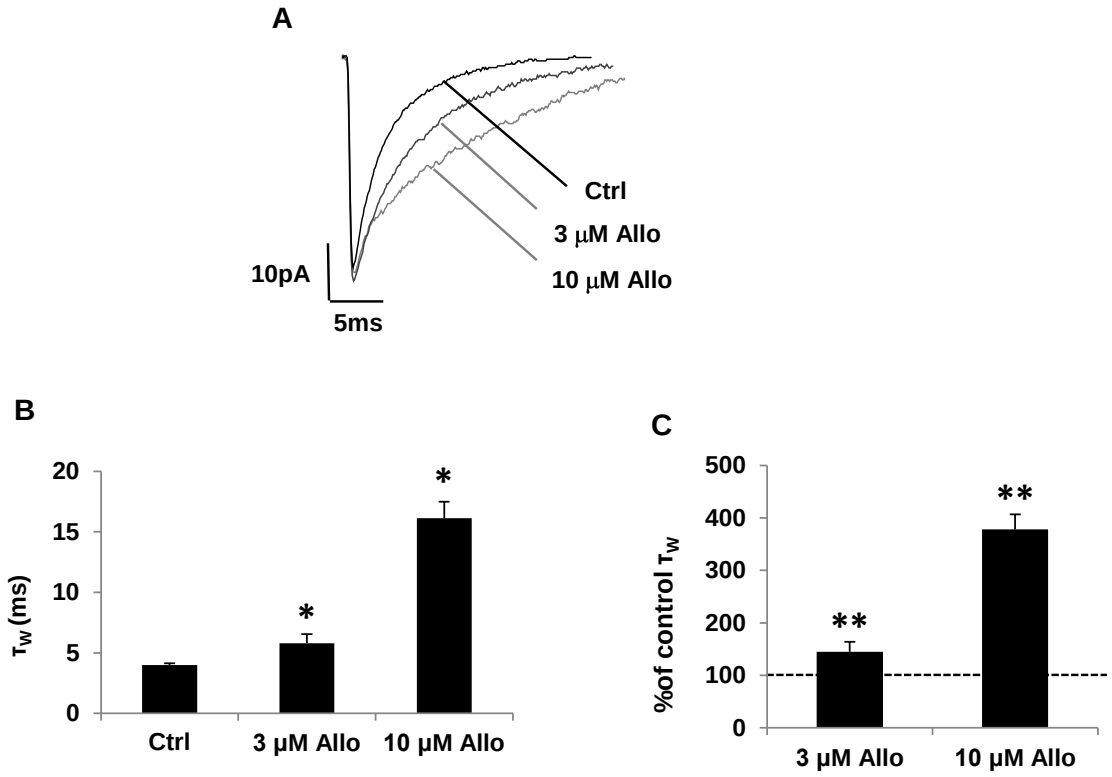

Figure 22 Intracellular allopregnanolone enhances synaptic $GABA_AR$ function of mature cortical neurones.

(A) Superimposed exemplar $GABA_AR$-mediated mIPSCs from a representative control mature L2/3 cortical neurone and from equivalent neurones treated with allopregnanolone (3 - 10 μM) administered intracellularly *via* the recording patch-electrode. Note recordings of mIPSCs for both control and steroid-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the steroid to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitudes of the mIPSCs are normalised to that of the control averaged mIPSC. **(B)** Histogram illustrating the concentration-dependent effect of intracellular allopregnanolone on the duration of the $GABA_AR$-mediated mIPSCs (time course of decay τ_w in ms; One-way ANOVA **P* <0.05; n = 6-7). **C.** Histogram illustrating the concentration-dependent effect of intracellular allopregnanolone on the duration of $GABA_AR$-mediated mIPSCs (τ_w expressed as a percentage of control 145 ± 19% and 378 ± 28%; n = 6-7; One-way RM ANOVA ***P* <0.05 *vs*. control. *Post hoc* Newman Keul's test revealed significant differences between the two concentrations of allopregnanolone, *P* <0.05). Allo = allopregnanolone; Ctrl = control.

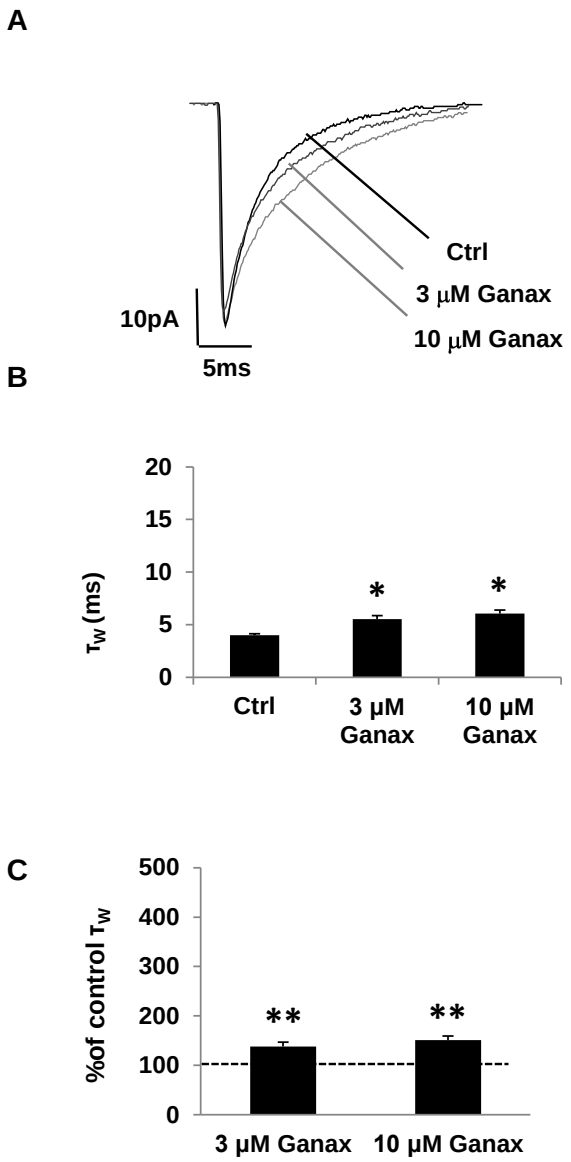

Figure 23 Intracellular ganaxolone enhances the synaptic $GABA_AR$ function of mature cortical neurones.

(A) Superimposed exemplar $GABA_AR$-mediated averaged mIPSCs from a representative control mature L2/3 cortical neurone and from equivalent neurones with 3 - 10 μM ganaxolone administered intracellularly *via* the recording patch-electrode. Note recordings of mIPSCs for both control and steroid-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the steroid to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(B)** Histogram illustrating the concentration-dependent effect of intracellular ganaxolone on the duration of $GABA_AR$-mediated mIPSCs (time course of decay τ_w in ms; One-way ANOVA $^*P$ <0.05; n = 6). **(C)** Histogram illustrating the effect of intracellular ganaxolone on the duration of $GABA_AR$-mediated mIPSCs (τ_w expressed as a percentage of control 138 ± 9% and 151 ± 8% n = 6; One-way RM ANOVA $^{**}P$ <0.05 *vs*. control *Post hoc* Newman Keul's test revealed no significant difference between the two concentrations of ganaxolone, P >0.05). Ganax = ganaxolone; Ctrl = control.

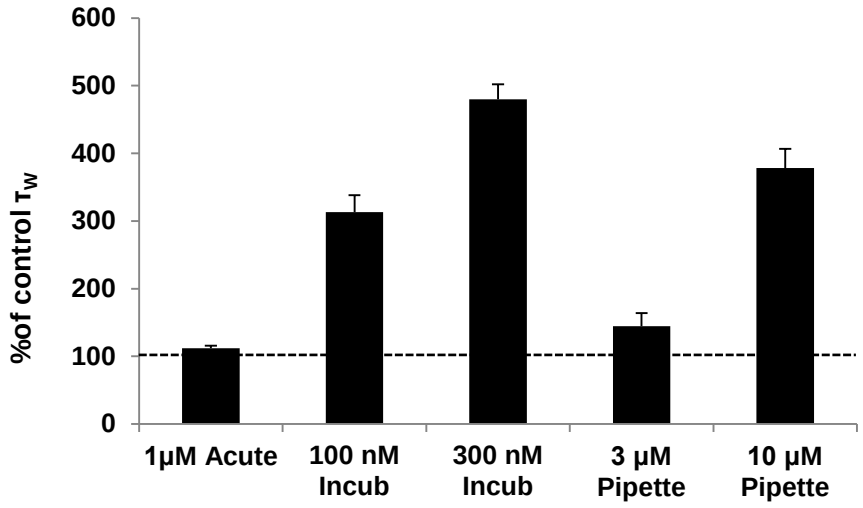

Figure 24 A comparison of the effects of extracellular acute, or prolonged and intracellular allopregnanolone on the function of synaptic $GABA_A$Rs of mature cortical neurones.

Histogram comparing the effectiveness of allopregnanolone administered by different methods to prolong $GABA_A$R-mediated mIPSCs (τ_w expressed as a percentage of control). In this regard allopregnanolone is clearly most effective when the brain slices are incubated with the drug for ~2 hours. Incub = ~2 hour incubation with allopregnanolone (n = 6-9). Pipette = steroid delivered intracellularly *via* the patch pipette. Acute = bath applied steroid with recordings commencing ~10 minutes after steroid perfusion.

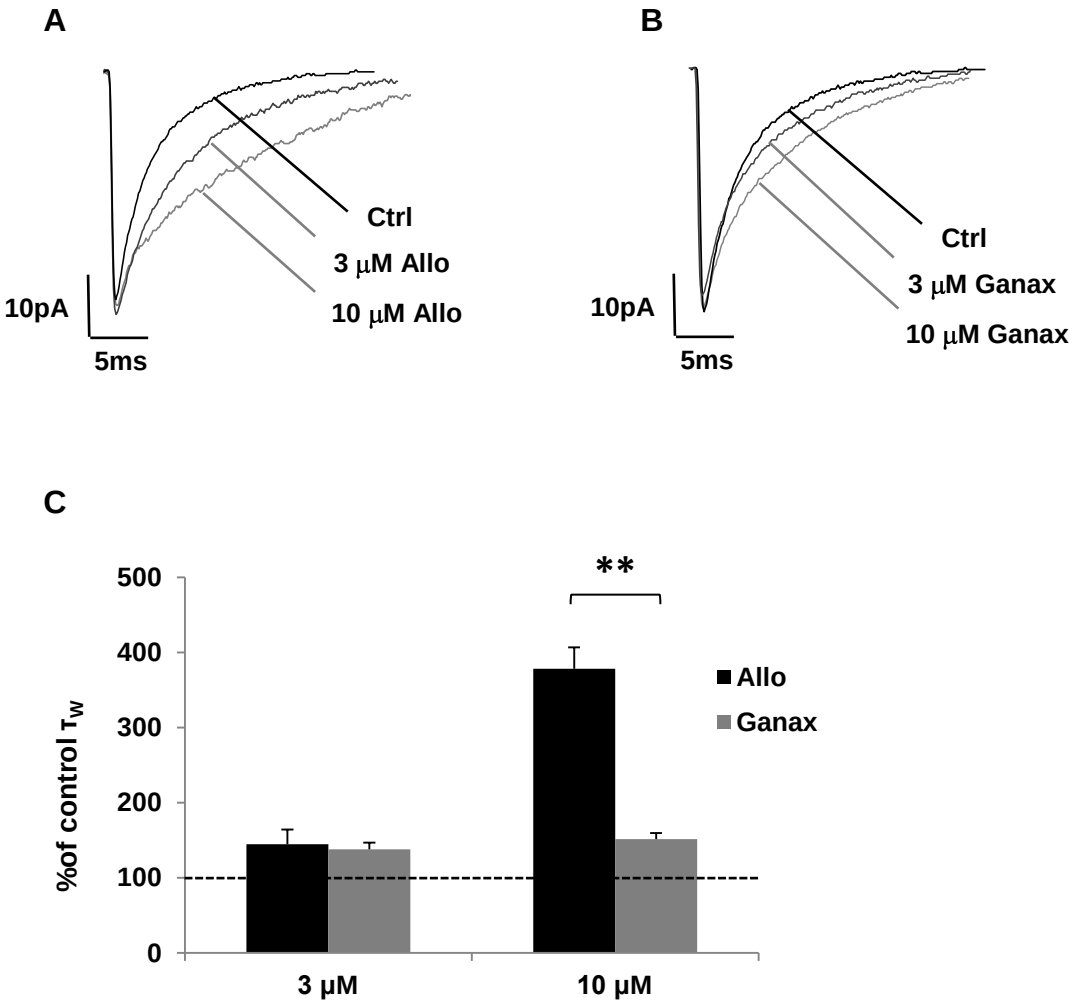

Figure 25 Comparison of the effects of intracellular allopregnanolone & ganaxolone on the function of synaptic $GABA_A$Rs of mature cortical neurones.

(A & **B)** Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs from representative control mature L2/3 cortical neurones and from equivalent neurones with 3 - 10 μM allopregnanolone, or ganaxolone administered intracellularly *via* the recording patch-electrode. **(C)** Histogram comparing the concentration-dependent effect of intracellularly allopregnanolone and ganaxolone on the duration of $GABA_A$R-mediated mIPSCs (time course of decay τ_w expressed as a percentage of control). The histogram illustrates the significantly greater effect of allopregnanolone compared to ganaxolone at the higher (10 μM) concentration: 3 μM (145 ± 19% *vs*. 138 ± 9%; $P > 0.05$; n = 6) and 10 μM (378 ± 28% *vs*. 151 ± 8%; n = 6-7 One-way RM ANOVA $^{**}P < 0.05$). Allo = allopregnanolone; Ganax = ganaxolone; Ctrl = control.

4.4 The effect of cyclodextrins on layer 2/3 cortical $GABA_A$ mIPSCs in mature WT mice.

As described in the Introduction, the cyclodextrins are relatively large barrel-shaped molecules that may be classified according to the number of glucose residues they contain (Brown, 2012; Shu *et al.,* 2004, 2007). The three principle types of cyclodextrins (CD) are the α-CD hexamer, the β-CD heptamer and the γ-CD octomer (Cooper *et al.,* 2005; Davis & Brewster, 2004; See Discussion: section 7.5 for further details). In the previous chapter, γ-CD was effective at revealing the presence of an endogenous neurosteroid tone that varied with development in nRT neurones, while in layer 2/3 cortical neurones it was discovered that the neurosteroid tone reappeared by P60-75. Whole-cell voltage-clamp recordings were made in layer 2/3 cortical pyramidal neurones with cyclodextrin present in the recording pipette (*i.e.* the optimal method of administration as described in the previous chapter). These recordings were compared against separate control recordings (*i.e.* they were not paired), where the recording pipette contained only the intracellular solution. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, charge transfer, decay kinetics and frequency as detailed in Table 16.

The $GABA_AR$ mIPSC decay (τ_W) of L2/3 cortical pyramidal neurones of WT mice was significantly decreased in the presence of γ-CD, but not by α-CD, or β-CD (τ_W control = 4.0 ± 0.1 ms, n = 35; γ-CD = 2.9 ± 0.1 ms, n = 15; α-CD = 4.1 ± 0.5 ms, n = 7; β-CD = 4.5 ± 0.4 ms, n = 6; One-way ANOVA, $P < 0.05$; Table 16, Figure 26). The T_{70} was similarly decreased in the presence of γ-CD but not by α-CD or by β-CD Table 16). γ-CD has been reported to have no direct effect on the $GABA_AR$ (Shu *et al.,* 2004, 2007) and the data reported has demonstrated that neither of the smaller molecules, α-CD or β-CD, had an impact on $GABA_AR$-mediated mIPSC decay time (See Discussion: section 7.5 for further details). These data agree with previous studies (Brown, 2012; Shu *et al.,* 2004, 2007), but the β-CD data potentially conflicts with Pytel *et al*., (2006) who reported that β-CD decreased the desensitisation kinetics of the $GABA_AR$ to GABA by direct modulation of the receptor itself within outside-out patches of cultured hippocampal neurones. The

mechanism for the direct modulation by β-CD was uncertain and the findings of Pytel *et al.,* (2006) have yet to be replicated.

The results are consistent with the hypothesis that γ-CD may shorten $GABA_AR$-mediated mIPSC decay time indirectly by the sequestration of endogenous neurosteroid compounds. A former laboratory colleague discovered that γ-CD had no effect on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones ~P20, indicating that endogenous neurosteroid tone is absent at that stage of development (Brown, 2012). The developmental data from the P20 cortical neurones serves as a control for my findings at P60-75.

Table 16 The effect of intracellular α/β/γ – cyclodextrin (CD) 0.5mM on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT α-CD (n=7)**	**WT β-CD (n=6)**	**WT γ-CD (n=15)**
Peak amp (pA)	-59 ± 2	-55 ± 3	-56 ± 3	-58 ± 2
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	-243 ± 15	-261 ± 16	**-185 ± 8
T70 (ms)	**5.2 ± 0.2**	**5.7 ± 0.6**	**5.9 ± 0.5**	****3.7 ± 0.2**
Tau w (ms)	**4.0 ± 0.1**	**4.1 ± 0.5**	**4.5 ± 0.4**	****2.9 ±0.1**
Frequency (Hz)	17 ± 2	18 ± 4	18 ± 5	18 ± 2

***P* < 0.05; one-way ANOVA, in comparison to age-matched control

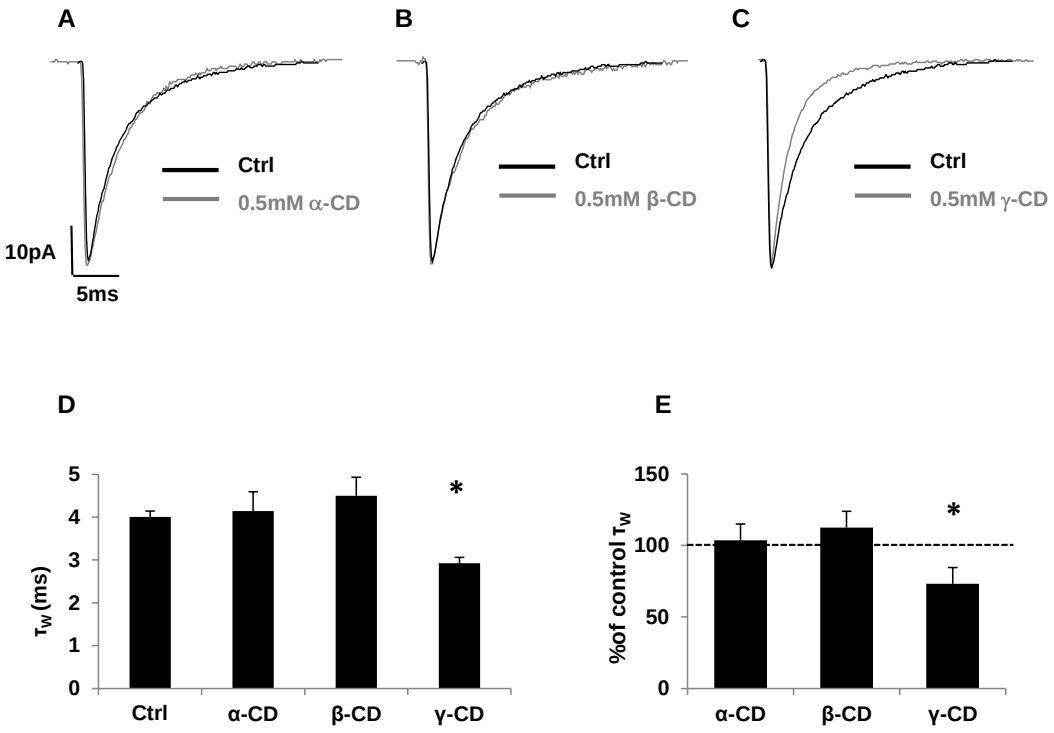

Figure 26 Intracellular γ-CD reduces the duration of $GABA_AR$-mediated mIPSCs of mature cortical neurones: evidence for an endogenous neurosteroid tone.

(A-C) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs from a representative control mature L2/3 cortical neurone and from equivalent neurones treated with 0.5 mM α-CD, β-CD or γ-CD administered intracellularly *via* the recording patch-pipette. Note recordings of mIPSCs for both control and cyclodextrin-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the compound to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. Note only γ-CD was effective in reducing the duration of the mIPSCs. **(D)** Histogram illustrating that intracellular γ-CD (but not α-CD or β-CD) decreases the $GABA_AR$ mIPSC τ_w (in ms) of mature L2/3 cortical neurones (One-way ANOVA $^*P < 0.05$; n = 6-15). **(E)** Histogram illustrating that intracellular γ-CD (but not α-CD or β-CD) decreases the $GABA_AR$-mediated mIPSC τ_w (expressed as a percentage of control) of mature L2/3 cortical neurones (73 ± 3%, 104 ± 11%, 112 ± 11% respectively *vs.* unpaired representative control n = 6-15; One-way RM ANOVA $^*P <$ 0.05). Dotted line indicates the control condition. Ctrl = control; γ-CD = γ-cyclodextrin (5 mM).

4.5 Is the effect of prolonged ganaxolone incubation on cortical $GABA_AR$-mediated mIPSCs influenced by intracellular γ-CD?

I have demonstrated that in mature L2/3 cortical neurones pipette-applied γ-CD can shorten the duration of mIPSCs, a finding consistent with the depletion of an endogenous neurosteroid tone. Furthermore, I have shown that prolonged ganaxolone incubation induces a marked prolongation of the mIPSC decay time. To probe whether the cyclodextrin effect is a consequence of neurosteroid sequestration I investigated whether intracellular γ-CD could reverse the ganaxolone-mediated modulation of the $GABA_AR$. Whole-cell voltage-clamp recordings were made in mature L2/3 cortical neurones of WT mice after at least two hours of incubation with ganaxolone with γ-CD applied in the recording pipette. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, decay kinetics, charge transfer and frequency (Table 17).

Pipette-applied γ-CD reduced significantly the effect of long incubation with ganaxolone, but did not reverse it completely (τ_W: control = 4.0 ± 0.1 ms, n = 35; γ-CD 0.5 mM = 2.9 ± 0.1 ms, n = 15; ganaxolone 300 nM = 10.9 ± 1 ms, n = 10; γ-CD 0.5 mM & ganaxolone 300 nM = 6.7 ± 0.9 ms, n = 7; One-way RM ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed a significant difference between ganaxolone alone and ganaxolone with γ-CD, $P < 0.05$; Table 17; Figure 27). The same effect on the mIPSC T_{70} was observed (Table 17). These results confirm that γ-CD is able to sequester neuroactive steroids such as ganaxolone. It appears that in the experiment described above, γ-CD may have become saturated by the relatively high concentrations of the neurosteroids in the brain slice tissue. These results support the proposal that the γ-CD effect observed for WT cortical neurones reflects the removal of an endogenous neurosteroid tone.

4.6 Can mature layer 2/3 cortical neurones synthesise neurosteroids?

As discussed above, progesterone and its' metabolite dihydroxy-progesterone (DHP) do not modulate $GABA_AR$s directly (Belelli & Herd, 2003; Brown, 2012), but require the activity of the enzymes 5α-R and 3α-HSD in order to synthesise allopregnanolone (Figure 3; Belelli & Lambert, 2005; Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003). Here the effects on mIPSCs of incubating the brain slices with the allopregnanolone precursors progesterone and DHP are investigated (Refer to neurosteroid synthesis pathway ; Figure 3).

Table 17 The effect of ganaxolone (Ganax) incubation treatment with γ-CD presented intracellularly, on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 300nM Ganax (n=10)**	**WT 300nM Ganax & 0.5mM γ-CD (n=7)**
Peak amplitude (pA)	-59 ± 2	-82 ± 6	**-62 ± 8
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-749 ± 88	**-418 ± 87
T70 (ms)	**5.2 ± 0.2**	**13.4 ± 1**	****9.4 ± 1**
Tau w (ms)	**4.0 ± 0.1**	**10.9 ± 1**	****6.7 ± 0.9**
Frequency (Hz)	17 ± 2	20 ± 3	14 ± 6

***P* < 0.05; One-way RM ANOVA (with *Post hoc* Newman Keul's test), in comparison to 300nM Ganaxolone

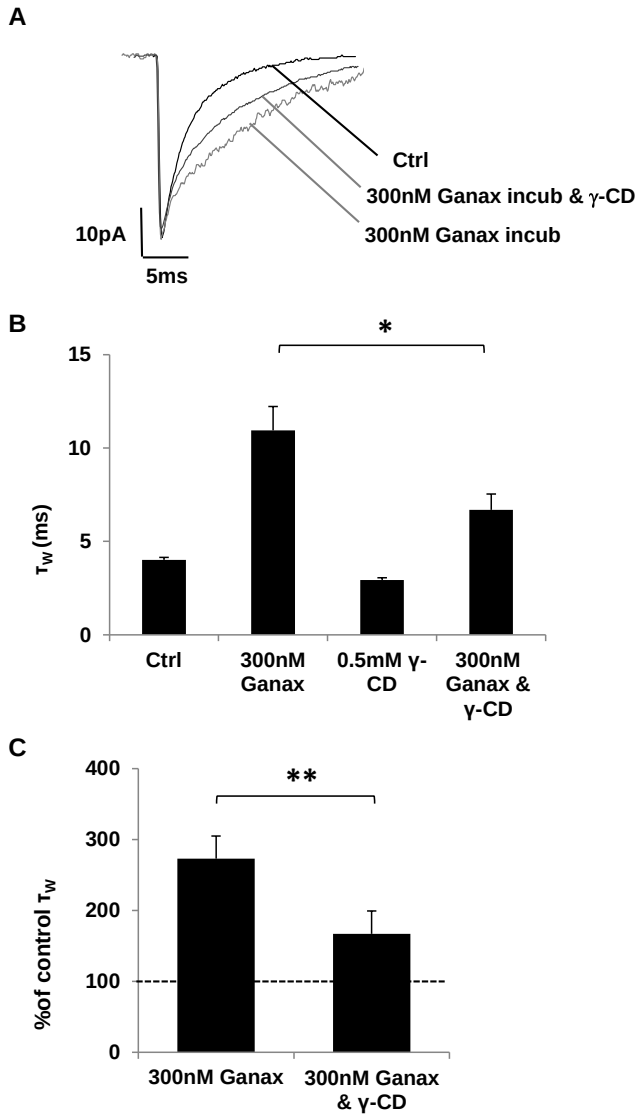

Figure 27 Intracellular γ-CD reduces the effect of extracellular ganaxolone on $GABA_A$R-mediated mIPSCs of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after pre-incubating the brain slice preparation with 300 nM ganaxolone (~2 hours), with, or without, intracellular 0.5 mM γ-CD. Note recordings of mIPSCs for both control and cyclodextrin-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the compound to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(B)** Histogram illustrating that intracellular 0.5 mM γ-CD significantly reduces the effectiveness of extracellular 300 nM ganaxolone to prolong the mIPSC decay (τ_w in ms; One-way RM ANOVA *P <0.05. *Post hoc* Newman Keul's test revealed a significant difference between ganaxolone alone and ganaxolone with γ-CD, P <0.05, n = 7-10. **(C)** Histogram illustrating that intracellular 0.5 mM γ-CD significantly reduces the effectiveness of 300 nM ganaxolone to prolong the mIPSC decay time (τ_w, expressed as a percentage of control 273 ± 32% *vs.* 167 ± 21%; n = 7-10 ; One-way RM ANOVA **P <0.05). Ganax = ganaxolone; Ctrl = control; γ-CD = γ-cyclodextrin; incub = ~ 2 hours pre-incubation.

4.7 The effect of prolonged progesterone incubation (1-50μM) on cortical $GABA_AR$-mediated mIPSCs of mature WT mice.

Whole-cell voltage-clamp recordings were made from L2/3 cortical neurones after at least two hours of pre-incubation of the preparation with progesterone. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, decay kinetics, charge transfer and frequency (Table 18). Progesterone produced a relatively modest prolongation of $GABA_AR$-mediated mIPSC decay time in WT mice. Interestingly, the highest concentration of progesterone tested was only slightly more effective than the lowest concentration investigated here (τ_W: control = 4.0 ± 0.1 ms, n = 35; progesterone 1 μM = 4.7 ± 0.3 ms, n = 6; progesterone 3 μM = 5.2 ± 0.2 ms, n = 8; progesterone 10 μM = 5.1 ± 0.3 ms, n = 9; progesterone 50 μM = 5.3 ± 0.2 ms, n = 9; One-way ANOVA, P <0.05; Table 18; Figure 28). Similarly, the mIPSC T_{70} was prolonged by progesterone incubation treatment (Table 18). These results suggest that the enzymatic function (5α-R and 3α-HSD) of mature L2/3 cortical neurones of WT mice is intact and that they are able to synthesise neurosteroids when their precursor (progesterone) is incubated with the brain slice preparation.

4.8 Does the 5α-reductase inhibitor finasteride block the effect of progesterone on the cortical mIPSCs of WT mice?

Finasteride itself has no direct effect on $GABA_AR$ mIPSCs, but pre-treatment with this enzyme inhibitor prevents the conversion of progesterone into $GABA_AR$- active neurosteroid metabolites in the hippocampal CA1 neurones of mature mice (Sanna *et al.,* 2004). Finasteride reduced the duration of synaptic GABAergic events in spinal LII neurones of immature rats, but had no effect on mature rats (Inquimbert *et al.,* 2007; Keller *et al.,* 2004). A similar phenomenon was observed in the cortical neurones of immature mice that were treated with another 5α-R inhibitor, SKF 105111 (Puia *et al.,* 2003). These findings are consistent with the hypothesis that there is a substantial endogenous neurosteroid tone in immature animals that has a significant modulatory effect on the $GABA_AR$ (Keller *et al.,* 2004).

Table 18 The effect of progesterone (Prog) incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 1μM Prog (n=6)**	**WT 3μM Prog (n=8)**	**WT 10μM Prog (n=9)**	**WT 50μM Prog (n=9)**	**WT 50μM Finast (n=7)**	**WT 50μM Prog & 50μM Finast (n=7)**
Peak amp (pA)	-59 ± 2	-65 ± 2	-64 ± 3	**-70 ± 2	**-91 ± 5	**-67 ± 3	-64 ± 3
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	**-319 ± 19	**-339 ± 26	**-373 ± 20	**-515 ± 34	**-299 ± 19	-269 ± 10
T70 (ms)	**5.2 ± 0.2**	****6.5 ± 0.5**	****6.8 ± 0.2**	****7.3 ± 0.4**	****7.6 ± 0.2**	**5.7 ± 0.2**	**5.7 ± 0.1**
Tau w (ms)	**4.0 ± 0.1**	****4.7 ± 0.3**	****5.2 ± 0.2**	****5.1 ± 0.3**	****5.3 ± 0.2**	**4.2 ± 0.1**	**4.1 ± 0.1**
Frequency (Hz)	17 ± 2	16 ± 2	17 ± 2	19 ± 2	**31 ± 3	**28 ± 5	19 ± 1.5

***P* < 0.05; One-way ANOVA in comparison to age-matched control, (with *Post hoc* Newman Keul's test).

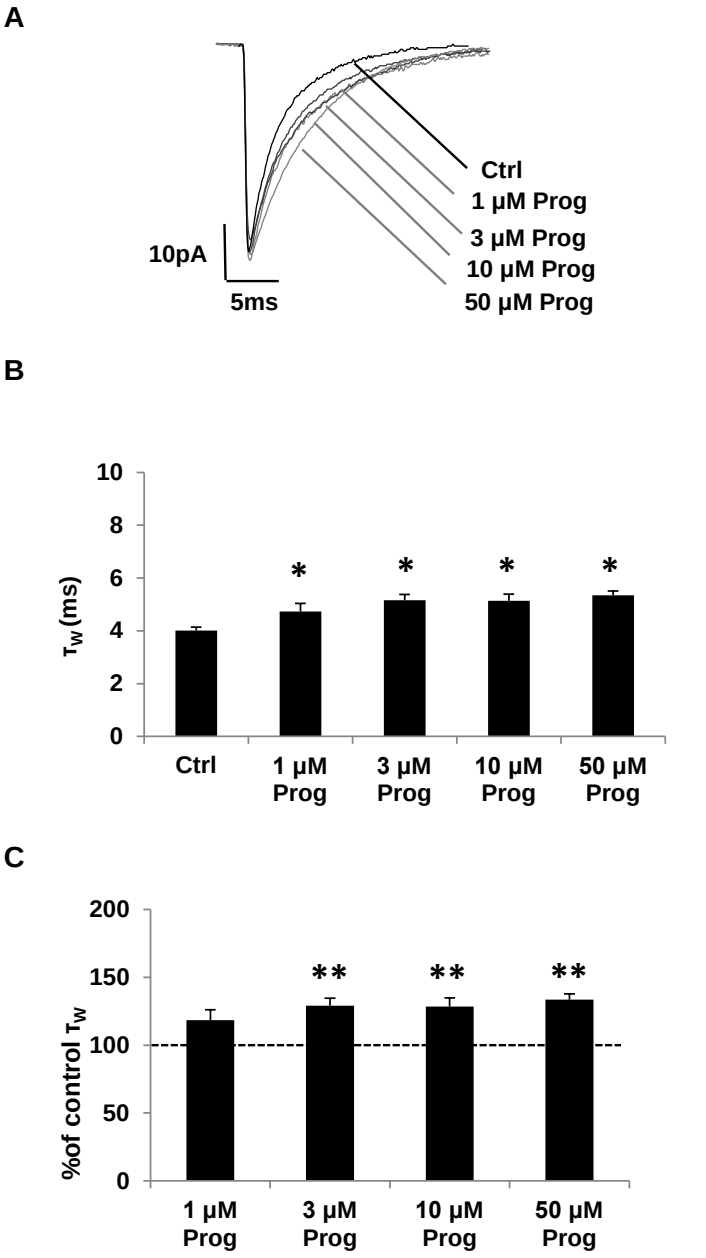

Figure 28 Prolonged exposure (~2 hrs) of mature cortical neurones to progesterone (1-50 μM) enhances the function of synaptic $GABA_A$Rs, suggesting that these neurones can synthesise allopregnanolone.

(A) Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after ~2 hour pre-incubation of the brain slice with 1 μM - 50 μM progesterone. **(B)** Histogram illustrating the significant, but modest effect of progesterone on duration of $GABA_A$R-mediated mIPSCs (decay time τ_w in ms;. One-way ANOVA *P <0.05. *Post hoc* Newman Keul's test revealed no significant inter-group difference between the concentrations of progesterone, P >0.05 n = 6-9). **(C)** Histogram illustrating the effect of progesterone (1 - 50 μM) on the duration of $GABA_A$R-mediated mIPSCs (τ_w expressed as a percentage of control). Progesterone increased τ_w (1 μM = 118 ± 8%, 3 μM = 129 ± 5%, 10 μM = 129 ± 6% and 30 μM = 133 ± 4% of control respectively; n = 6-9; One-way RM ANOVA **P >0.05). Note the dotted line indicates the control condition. Ctrl = control; Prog = progesterone.

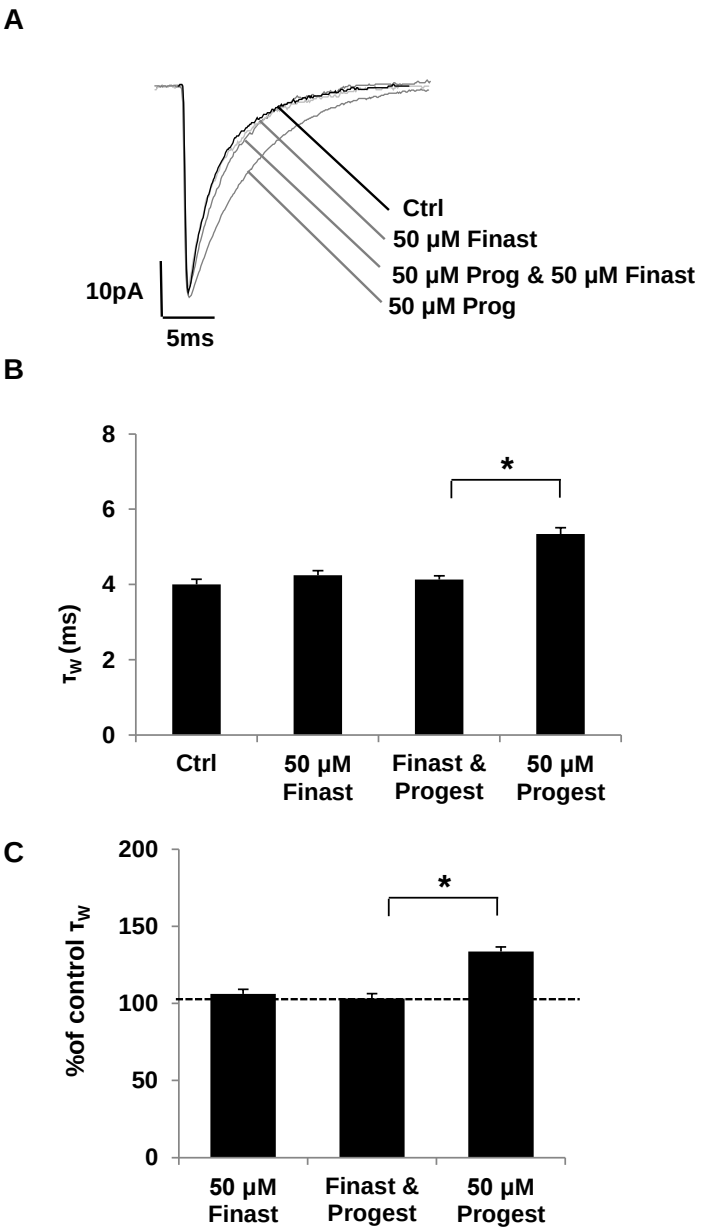

Figure 29 The 5α-reductase inhibitor finasteride prevents the effects of progesterone incubation on synaptic $GABA_A$Rs of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after ~2 hour pre-incubation of the brain slice with either 50 µM finasteride alone, or co-applied with 50 µM progesterone. **(B)** Histogram illustrating that finasteride prevents the effect of progesterone to prolong the $GABA_A$R-mediated mIPSC τ_w (in ms); One-way ANOVA P <0.05. *Post hoc* Newman Keul's test revealed a significant difference for progesterone alone *c.f.* when progesterone is co-applied with finasteride *P <0.05; n = 7-9 X). **(C)** Histogram illustrating that finasteride prevents the effect of progesterone to prolong $GABA_A$R-mediated mIPSCs (τ_w expressed as a percentage of control: 106 ± 3%, 103 ± 3% and 133 ± 4% respectively, n = 7-9; One-way RM ANOVA P <0.05. *Post hoc* Newman Keul's test revealed differences for progesterone 50 µM with or without finasteride 50 µM, **P <0.05). Ctrl. = control; Prog = progesterone; Finast = finasteride.

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones of WT mice after at least two hours of incubation with finasteride and progesterone. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 18). Finasteride alone had no effect on L2/3 cortical neurone $GABA_AR$ mIPSC decay time in WT mice, but it did prevent the effect of progesterone (τ_W: control = 4.0 ± 0.1 ms, n = 35; finasteride 50 µM = 4.2 ± 0.1 ms, n = 7; progesterone 50 µM = 5.3 ± 0.2 ms, n = 9; finasteride 50 µM & progesterone 50 µM = 4.1 ± 0.1 ms, n = 7; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed differences for progesterone 50 µM with, or without, finasteride (50 µM, P <0.05; Table 18; Figure 29). Similarly, the mIPSC T_{70} was unaffected by finasteride, but this enzyme inhibitor prevented the effect of progesterone (Table 18). These results indicate that progesterone requires the enzymatic activity of 5α-R for it to be converted to its' active metabolites. Of interest, the observation that finasteride alone had no effect on $GABA_AR$ mIPSC decay time, despite the suggested presence of a modest endogenous neurosteroid tone in mature WT mice, was made. This apparent paradox may be explained by comparing how finasteride and CD act. CD will remove the endogenous neurosteroid present, whereas although finasteride should prevent new allopregnanolone synthesis, it will have little impact on that already present. Therefore, the apparent lack of an effect of finasteride may reflect the relatively slow turnover of pre-synthesised allopregnanolone.

4.9 The effect of acute DHP & prolonged DHP incubation on cortical $GABA_AR$ mIPSCs of mature WT mice.

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones before and after the bath application of DHP. The neurosteroid was bath-applied after at least four minutes of stable recording enabling the comparison of paired recordings. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, decay kinetics, charge transfer and frequency (Table 19). After ten minutes application, DHP (3µM) had no effect on the properties of

synaptic $GABA_AR$ events in WT layer 2/3 cortical neurones (τ_W: Control = 5 ± 0.3 ms, n = 4; 3 μM DHP = 4.7 ± 0.3 ms, n = 4; Student's paired t test, $P > 0.05$). The T_{70} was similarly little influenced by this steroid; Table 19; Figure 30). These findings are consistent with work carried out by a former laboratory colleague in ventrobasal neurones of the thalamus in the earlier stages of development (Brown, 2012). The lack of effect after the acute application of DHP contrasts to the clear, albeit modest, effects of acutely applied allopregnanolone and ganaxolone. Given the lack of effect of acute DHP, I investigated whether prolonged incubation with this allopregnanolone precursor was effective in prolonging $GABA_AR$-mediated mIPSCs.

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones after at least two hours of brain slice incubation with DHP. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, decay kinetics, charge transfer and frequency (Table 20). Two hours of brain slice incubation with DHP (1 – 3 μM) produced a significant, concentration-dependent prolongation of layer 2/3 cortical $GABA_AR$-mediated mIPSC decay time in WT mice (τ_W: control = 4.0 ± 0.1 ms, n = 35; DHP 1 μM = 5.9 ± 0.2ms, n = 14; DHP 3 μM = 10.2 ± 0.1 ms, n = 9; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed intergroup differences between all three treatments, $P < 0.05$; Table 20; Figure 30). Similarly, the mIPSC T_{70} was significantly prolonged by DHP (Table 20). These results indicate that mature WT L2/3 cortical neurones have intact 3α-HSD enzymatic function and are able to convert DHP into the active metabolite allopregnanolone.

Table 19 The effect of DHP applied acutely *via* the intracellular or extracellular routes on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 3μM DHP in pipette (n = 6)**	**WT 10μM DHP in pipette (n = 6)**	**WT Pre 3μM DHP in ECS (n=4)**	**WT Post 3μM DHP In ECS (n=4)**
Peak amplitude (pA)	-59 ± 2	-55 ± 2	-67 ± 4	-60 ± 4	-61 ± 2
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-229 ± 14	**-302 ± 12	-302 ± 4	-292 ± 8
T70 (ms)	**5.2 ± 0.2**	**5.3 ± 0.2**	**5.5 ± 0.4**	**6.6 ± 0.6**	**6.3 ± 0.6**
Tau w (ms)	**4.0 ± 0.1**	**3.9 ± 0.1**	**4.3 ± 0.3**	**5.0 ± 0.3**	**4.7 ± 0.3**
Frequency (Hz)	17 ± 2	22 ± 1	18 ± 2	19 ± 3	20 ± 6

**$P < 0.05$; One-way ANOVA, in comparison to age-matched control

Table 20 The effect of DHP incubation treatment (with γ-CD presented intracellularly), on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 1μM DHP (n=14)**	**WT 3μM DHP (n=9)**	**WT 3μM DHP & 0.5mM γ-CD (n=5)**
Peak amp (pA)	-59 ± 2	**-73 ± 4	**-69 ± 5	-73 ± 10
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ±0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	**-433 ± 31	**-646 ± 33	-552 ± 80
T70 (ms)	**5.2 ± 0.2**	****8 ± 0.4**	****13.9 ± 1**	***9.9 ± 0.4**
Tau w (ms)	**4.0 ± 0.1**	****5.9 ± 0.2**	****10.2 ± 1**	***7.3 ± 0.1**
Frequency (Hz)	17 ± 2	16 ± 2	19 ± 6	12 ± 2

*$P < 0.05$; One-way RM ANOVA in comparison to 3μM DHP (with *Post hoc* Newman Keul's test).

**$P < 0.05$; One-way ANOVA in comparison to control (with *Post hoc* Newman Keul's test).

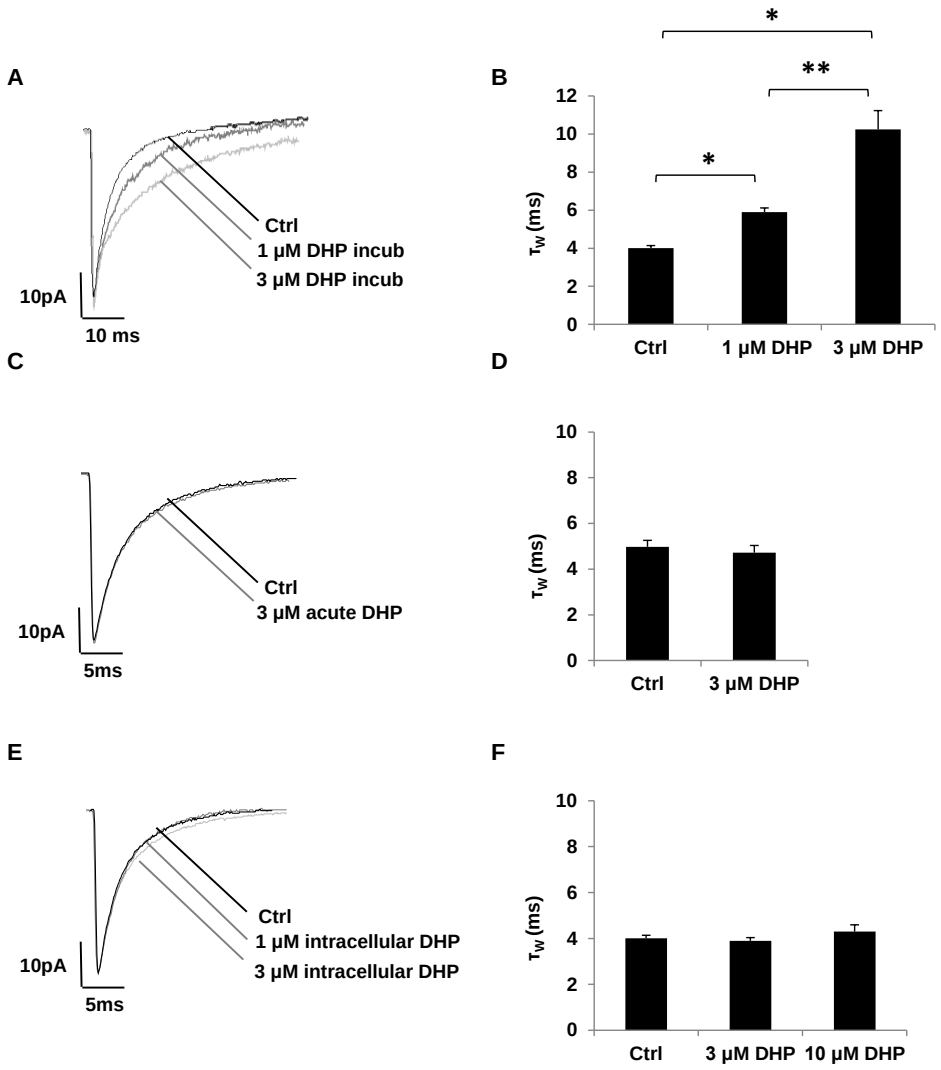

Figure 30 Prolonged exposure (~2 hrs) of mature cortical neurones to DHP greatly enhances the function of synaptic $GABA_A$Rs, but acute extracellular or intracellular DHP is inert.

(A) Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after an ~2 hour pre-incubation of the brain slice with 1 - 3 μM DHP. **(B)** Histogram illustrating the considerable concentration-dependent effect of DHP on the duration of $GABA_A$R-mediated mIPSCs (τ_w in ms; One-way ANOVA *P <0.05. Post hoc analysis revealed intergroup differences between 1 μM and 3 μM n = 9-14, Newman Keuls **P <0.05). **(C)** Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a mature cortical neurone before and after acute exposure to 3μM DHP. **(D)** Histogram illustrating the lack of effect of acutely applied 3μM DHP on $GABA_A$R-mediated mIPSCs (τ_w in ms, Student's paired t test P >0.05 *vs.* control n = 4). **(E)** Superimposed exemplar averaged $GABA_A$R-mediated mIPSCs acquired from a representative control cortical neurone and from equivalent neurones treated with DHP (3 - 10 μM) administered intracellularly *via* the recording patch-electrode. Note recordings of mIPSCs for both control and steroid-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the steroid to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(F)** A histogram illustrating the lack of effect of intracellular DHP on the duration of $GABA_A$R-mediated mIPSCs (τ_w in ms, n = 6, One-way ANOVA P >0.05 *vs.* control). Ctrl = control; DHP = dihydroxyprogesterone, incub = incubation.

4.10 Does intracellular cyclodextrin influence the effect of pre-incubated DHP on cortical mIPSCs of WT mice?

I have previously demonstrated that pipette-applied γ-CD can reduce the duration of cortical mIPSCs (see Sections 3.10 & 4.4) an effect interpreted as occurring as a consequence of removing an endogenous neurosteroid tone. In support of this, intracellular γ-CD reduced the effect of ganaxolone on mIPSCs. DHP incubation treatment of brain slice tissue induces a marked prolongation of $GABA_AR$ mIPSC decay time, which raised the question of whether pipette-applied γ-CD could reduce the modulatory effect of DHP incubation as it had done with ganaxolone. Whole-cell voltage-clamp recordings were made in mature L2/3 cortical neurones of WT mice after at least two hours of incubation with DHP with γ-CD applied in the recording pipette. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 20.).

Pipette-applied γ-CD reduced the effect of incubation with DHP (τ_W: control = 4.0 ± 0.1 ms, n = 35; γ-CD 0.5 mM = 2.9 ± 0.1 ms, n = 15; DHP 3 μM = 10.2 ± 1 ms, n = 9; γ-CD 0.5 mM & DHP 3 μM = 7.3 ± 0.1 ms, n = 5; One-way RM ANOVA, P <0.05; *post hoc* Newman Keul's test revealed a significant difference between DHP alone and DHP with γ-CD, P <0.05; Table 20; Figure 31). The same effect on the mIPSC T_{70} was observed (Table 20). These results confirm that γ-CD is also able to sequester the GABA-active neurosteroid metabolite of DHP. In common with the ganaxolone experiments, γ-CD did not fully reverse the effect of DHP suggesting that γ-CD may have become saturated by the relatively high concentrations of the neurosteroids in the brain slice tissue.

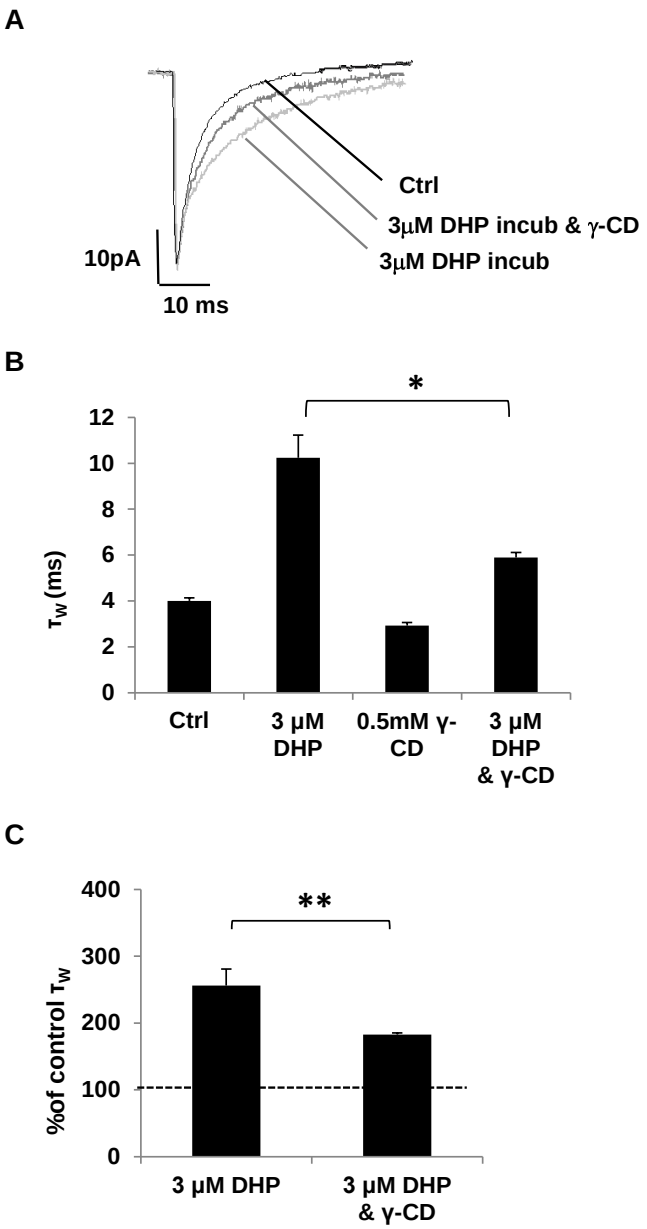

Figure 31 Intracellular γ-CD reduces the effect of DHP incubation on $GABA_AR$ mIPSCs of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs from a representative control mature L2/3 cortical neurone and from equivalent neurones after 3 μM DHP (~2 hour) pre-incubation or 3 μM DHP pre-incubation with 0.5 mM γ-CD administered intracellularly *via* the recording patch-electrode. Note recordings of mIPSCs for both control and cyclodextrin-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the steroid scavenger to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(B)** Histogram illustrating that intracellular 0.5 mM γ-CD significantly reduces the effect of 3 μM DHP pre-incubation to prolong the mIPSC duration (τ_w in ms; One-way ANOVA $^*P$ <0.05; n = 5-9). **(C)** Histogram illustrating that intracellular 0.5 mM γ-CD significantly reduces the effect of 3 μM DHP pre-incubation on the mIPSC duration (τ_w expressed as a percentage of control 256 ± 25% *vs.* 183 ± 3% n = 5-9 (one-way RM ANOVA $^{**}P$ <0.05). Ctrl = control; DHP = dihydroxyprogesterone, γ-CD = γ-cyclodextrin, incub = incubation.

4.11 The effect of intracellular DHP on layer 2/3 cortical $GABA_A$-mediated mIPSCs of mature WT mice.

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones with DHP present in the recording pipette. These recordings were compared to a separate set of control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 19). Pipette-applied DHP (3 μM - 10 μM) had no effect on the properties of $GABA_AR$ mIPSCs of WT layer 2/3 cortical neurones (τ_W control = 4.0 ± 0.1 ms, n = 35, 3 μM DHP = 3.9 ± 0.1 ms, n = 6; 10 μM DHP = 4.3 ± 0.3 ms, n = 6; One-way ANOVA, $P > 0.05$; Table 19; Figure 30). The T_{70} was similarly unaffected by the presence of DHP (Table 19). These results contrast with the effects of pipette-applied allopregnanolone and ganaxolone described above (Figure 32). The lack of effect of intracellular DHP suggests that the neurone being recorded from cannot synthesise allopregnanolone either because the neurone is not a site of synthesis, or more probably that pipette dialysis has compromised neurosteroid synthesis.

4.12 Provera, an inhibitor of 3α-HSD, prevents the effects of DHP but not ganaxolone on cortical mIPSCs of mature WT mice.

As described previously, provera inhibits 3α-HSD, consequently preventing the conversion of DHP to allopregnanolone (Belelli & Herd, 2003; Belelli & Lambert, 2005; Sunde *et al.,* 1982). Provera is also known to modulate $GABA_AR$ mIPSCs in dentate gyrus neurones, perhaps as a consequence of the differential expression of different isoforms of 3α-HSD in these neurones (Belelli & Herd, 2003). Whole-cell voltage-clamp recordings were made in mature layer 2/3 cortical neurones of wild type mice after at least two hours of incubation with provera and DHP, or provera and ganaxolone. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 21).

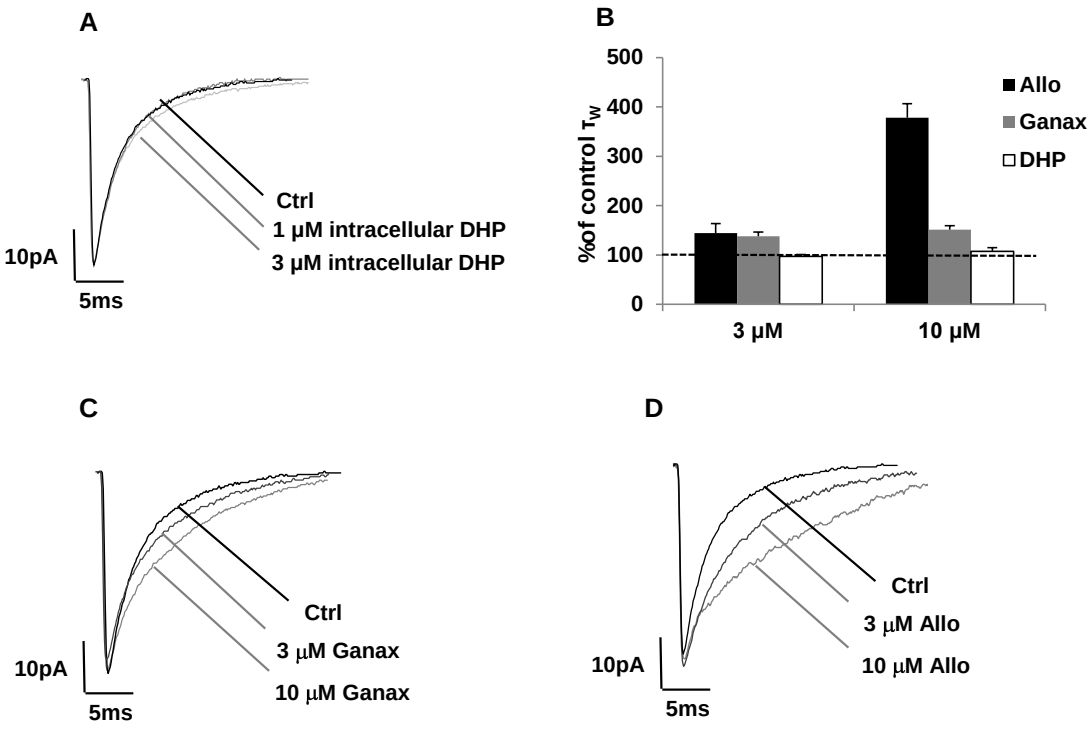

Figure 32 By contrast to allopregnanolone and ganaxolone, intracellular DHP has no effect on the function of synaptic $GABA_AR$s of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs acquired from a control cortical neurone and from equivalent neurones with 3 - 10 μM DHP administered intracellularly *via* the recording patch-electrode. Note recordings of mIPSCs for both control and steroid-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the steroid to equilibrate (Evans & Marty, 1986). To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC. **(B)** Histogram illustrating that intracellular DHP (3 - 10 μM) has no effect on the duration of $GABA_AR$-mediated mIPSCs (τ_w expressed as a percentage of control; one-way RM ANOVA P >0.05; n = 6), by contrast to allopregnanolone (3 – 10 μM) and ganaxolone (3 - 10μM). The dotted line indicates control. **(C** & **D)** Superimposed exemplar averaged $GABA_AR$-mediated mIPSC acquired from a representative control cortical neurone and from equivalent neurones internally dialysed with allopregnanolone (3 - 10 μM), or ganaxolone (3 - 10 μM). Ctrl = control; Allo = allopregnanolone; Ganax = ganaxolone; DHP = dihydroxyprogesterone

Table 21 The 3α-HSD inhibitor provera reduces the effect of DHP but not ganaxolone (Ganax) incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 1uM Provera (n=6)**	**WT 3μM DHP (n=9)**	**WT Provera & 3uM DHP (n=7)**	**WT 300nM Ganax (n=10)**	**WT Provera & 300nM Ganax (n=7)**
Peak amplitude (pA)	-59 ± 2	-66 ± 5	-69 ± 5	-61 ± 3	-82 ± 6	-75 ± 4
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ±0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-310 ± 23	-646 ± 33	**-327 ± 19	-749 ± 88	-778 ± 46
T70 (ms)	**5.2 ± 0.2**	**6.3 ± 0.3**	**13.9 ± 1**	**** 6.9 ± 0.1**	**13.4 ± 1**	**15.5 ± 0.7**
Tau w (ms)	**4.0 ± 0.1**	**4.6 ± 0.2**	**10.2 ± 1**	**** 5.2 ± 0.1**	**10.9 ± 1**	**10.9 ± 0.7**
Frequency (Hz)	17 ± 2	23 ± 4	19 ± 6	16 ± 3	20 ± 3	17 ± 3

**$P < 0.05$; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to 3μM DHP

Provera alone exerted a modest effect on L2/3 cortical neurone $GABA_AR$ mIPSC decay time but, in addition, it prevented the effect of DHP (τ_W: control = 4.0 ± 0.1 ms, n = 35; provera 1 μM = 4.6 ± 0.2 ms, n = 6; DHP 3 μM = 10.2 ± 1 ms, n = 9; provera 1 μM & DHP 3 μM = 5.2 ± 0.1 ms, n = 7; One-way ANOVA, *P* <0.05; *post hoc* Newman Keul's test revealed differences for DHP 3 μM with or without provera 1 μM, *P* <0.05; Table 21; Figure 33). The same effect on the mIPSC T_{70} was observed (Table 21).

As anticipated, provera did not inhibit the effect of ganaxolone on $GABA_AR$ mIPSCs in mature L2/3 cortical neurones of WT mice (τ_W: control = 4.0 ± 0.1 ms, n = 35; provera 1 μM = 4.6 ± 0.2 ms, n = 6; ganaxolone 300 nM = 10.9 ± 1 ms, n = 10; provera 1 μM & ganaxolone 300 nM = 10.9 ± 0.7 ms, n = 7; One-way ANOVA, *P* <0.05; *post hoc* Newman Keul's test revealed no differences for ganaxolone 300 nM with, or without, provera 1 μM, *P* >0.05; Table 21; Figure 33). The same effect on the mIPSC T_{70} was observed (Table 21). These results confirm that DHP requires enzymatic conversion by 3α-HSD for it to induce a prolongation in the decay time of $GABA_AR$ mIPSCs. In contrast, ganaxolone was unaffected by 3α-HSD inhibition with provera.

4.13 The 3α-HSD inhibitor indometacin prevents the effect of DHP but not allopregnanolone on cortical mIPSCs of mature WT mice.

Indometacin has an entirely different molecular structure to provera, but in common it is also an effective inhibitor of the enzyme 3α-HSD (Askonas *et al.,* 1991; Belelli & Herd, 2003; Belelli & Lambert, 2005; Duax *et al.,* 1978; Hori *et al.,* 2006; Sunde *et al.,* 1982). Whole-cell voltage-clamp recordings were made in mature L2/3 cortical neurones of wild type mice after at least two hours of incubation with indometacin and DHP. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 22).

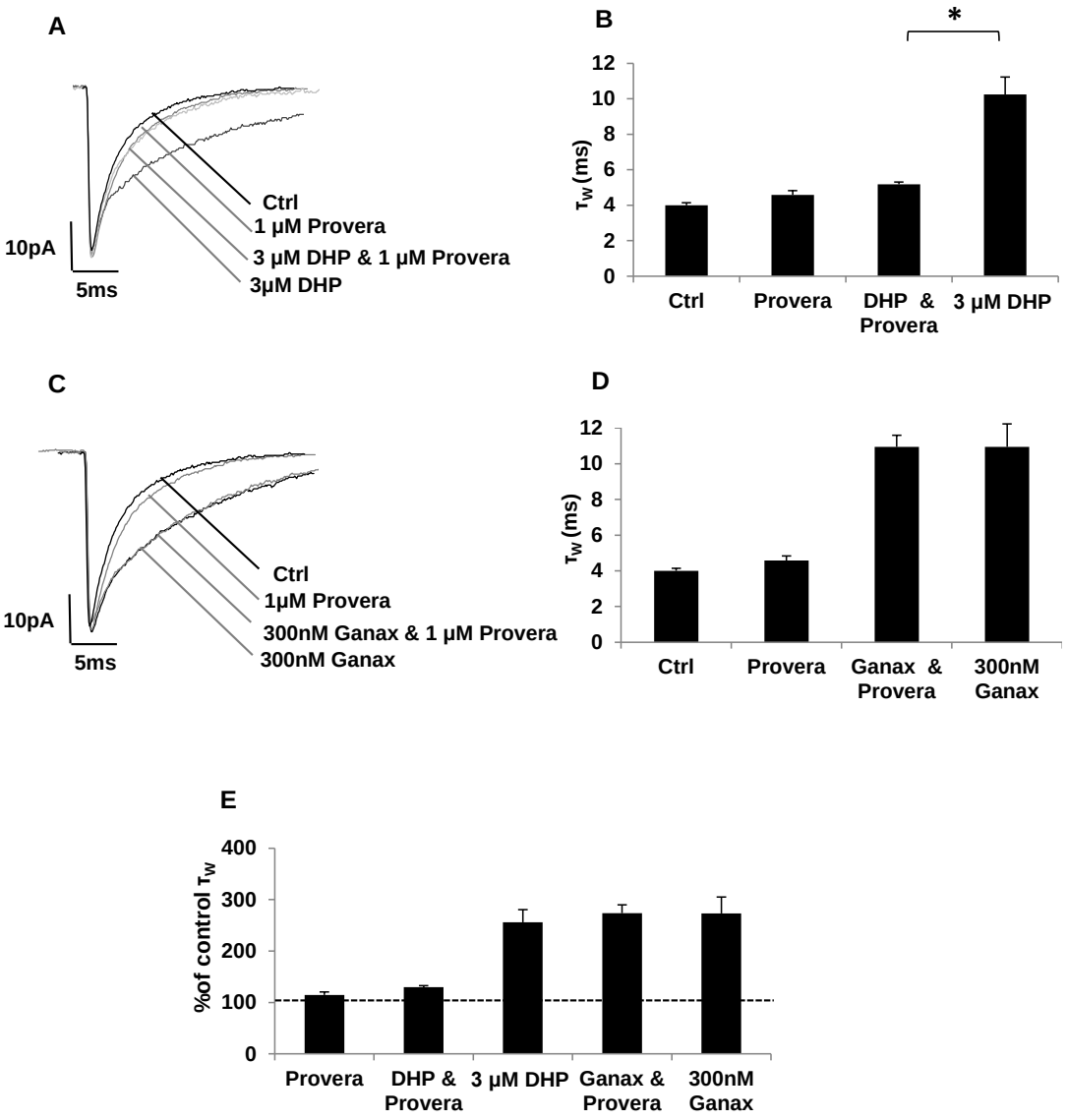

Figure 33 The 3α-HSD enzyme inhibitor provera suppresses the effects of DHP, but not ganaxolone on $GABA_AR$-mediated mIPSCs of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs from a representative control cortical neurone and from equivalent neurones after an ~2 hour pre-incubation of the brain slice with provera (1 μM), DHP (3 μM), or both. To facilitate comparison of their kinetics, the amplitude of the mIPSCs are normalised to that of the control averaged mIPSC **(B)** Histogram illustrating that provera prevents the effect of DHP pre-incubation to prolong $GABA_AR$-mediated mIPSCs (τ_w in ms; one-way ANOVA P <0.05. *Post hoc* Newman Keul's test revealed differences for DHP (3 μM) with or without provera (1 μM), *P <0.05, n = 7-9). **(C)** Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs from a representative control cortical neurone and from equivalent neurones after an ~2 hour pre-incubation of the brain slice with provera (1 μM), ganaxolone (300 nM), or both. **(D)** Histogram illustrating that provera did not prevent the effect of ganaxolone on the duration of $GABA_AR$-mediated mIPSCs (τ_w in ms; one-way ANOVA P <0.05. *Post hoc* Newman Keul's test revealed no difference for the effects of ganaxolone (300 nM) with, or without provera (1 μM), P >0.05, n = 7-10). **(E)** Histogram illustrating the selective effect of the 3α-HSD enzyme inhibitor provera to suppress the effect of DHP on the mIPSC τ_w (expressed as a percentage of control), but not that of ganaxolone. Ctrl = control; DHP = dihydroxyprogesterone

Table 22 The 3α-HSD inhibitor indometacin (indo) reduces the effect of DHP incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice

	WT Control (n=35)	**WT 30µM Indo (n=6)**	**WT 3µM DHP (n=9)**	**WT 3µM DHP & 30µM Indo (n=6)**
Peak amplitude (pA)	-59 ± 2	-53 ± 2	-69 ± 5	**-56 ± 4
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ±0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	-222 ± 6	-646 ± 33	**-263 ± 23
T70 (ms)	**5.2 ± 0.2**	**5.1 ± 0.2**	**13.9 ± 1**	****5.8 ± 0.3**
Tau w (ms)	**4.0 ± 0.1**	**4.6 ± 0.2**	**10.2 ± 1**	** **5.0 ± 0.3**
Frequency (Hz)	17 ± 2	14 ± 2	19 ± 6	14 ± 2

***P* < 0.05; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to 3µM DHP

Indometacin alone had no effect on the properties of $GABA_AR$ mIPSCs, including decay time, but it prevented the effect of DHP (τ_W: control = 4.0 ± 0.1 ms, n = 35; indometacin 30 μM = 4.6 ± 0.2 ms, n = 6; DHP 3 μM = 10.2 ± 1 ms, n = 9; indometacin 30 μM & DHP 3 μM = 5 ± 0.3 ms, n = 6; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed differences for DHP 3 μM with or without indometacin 100 μM, P <0.05; Table 22; Figure 34). The same effect on the mIPSC T_{70} was observed (Table 22).

By contrast, indometacin had no effect on the prolongation of mIPSCs by pre-incubated allopregnanolone (τ_W: control = 4.0 ± 0.1 ms, n = 35; indometacin 100 μM = 4.2 ± 0.1 ms, n = 7; allopregnanolone 100 nM = 12.5 ± 1 ms, n = 9; indometacin 100 μM & allopregnanolone 100 nM = 13.5 ± 0.8ms, n = 7; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed no differences for allopregnanolone 100 nM with or without indometacin 100 μM, P >0.05; Table 23; Figure 34). The same effect on the mIPSC T_{70} was observed (Table 23). These results confirm that allopregnanolone does not require 3α-HSD in order to modulate $GABA_AR$ mIPSCs in mature L2/3 cortical neurones. It is interesting that indometacin did not increase the effect of allopregnanolone by potentially inhibiting its metabolism to an inactive form. These experiments with another 3α-HSD enzyme inhibitor confirm that DHP requires conversion to a more active form by 3α-HSD in order for it to induce prolongation of $GABA_AR$ mIPSC decay time.

In contrast to the findings with allopregnanolone, indometacin exhibited concentration-dependent inhibition of ganaxolone incubation treatment (τ_W: control = 4.0 ± 0.1 ms, n = 35; indometacin 30 μM = 4.6 ± 0.2 ms, n = 6; indometacin 100 μM = 4.6 ± 0.1 ms, n = 7; ganaxolone 300 nM = 10.9 ± 1 ms, n = 10; indometacin 30 μM & ganaxolone 300 nM =6.2 ± 0.5 ms, n = 10; indometacin 100 μM & ganaxolone 300 nM = 5.4 ± 0.3 ms, n = 8; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed significant differences between three of the treatment groups: ganaxolone 300nM alone, ganaxolone 300nM with indometacin 30 μM and ganaxolone 300nM with indometacin 100 μM, P <0.05; Table 23; Figure 34). The same effect on the mIPSC T_{70} was observed (Table 23).

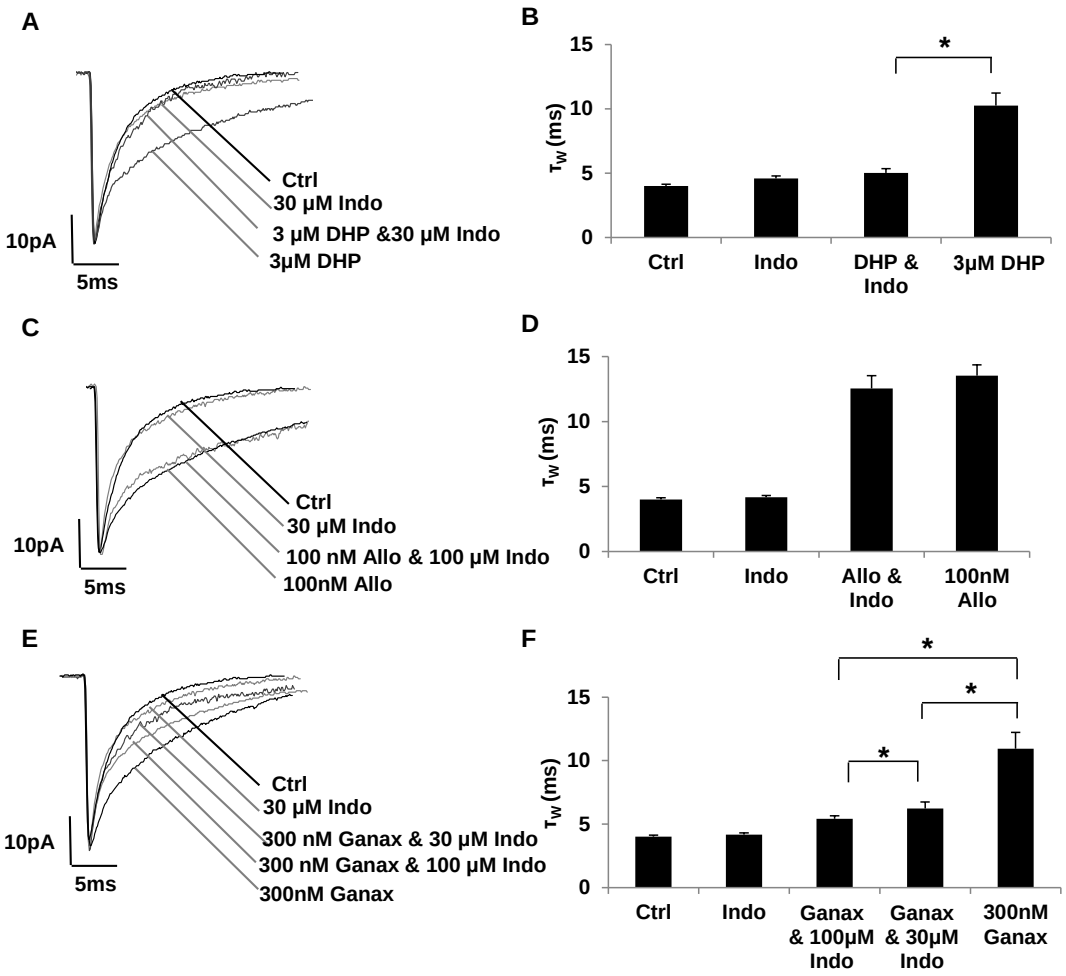

Figure 34 The 3α-HSD enzyme inhibitor indometacin suppresses the effects of DHP and ganaxolone, but not those of allopregnanolone on $GABA_AR$-mediated mIPSCs of mature cortical neurones.

(A) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs acquired from a representative control cortical neurone and from equivalent neurones after ~2 hours pre-incubation of the brain slice with indometacin (30 μM), DHP (3 μM), or both. **(B)** Histogram illustrating that indometacin prevents the effect of DHP on the duration of the $GABA_AR$-mediated mIPSCs (τ_w in ms, one-way ANOVA P <0.05. *Post hoc* Newman Keul's test revealed differences for DHP 3 μM with or without indometacin 100 μM, *P <0.05, n = 6-9). **(C)** Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs acquired from a representative control neurone and from equivalent neurones after an ~2 hour pre-incubation of the brain slice with indometacin (100 μM), allopregnanolone (100 nM), or both. **(D)** Histogram illustrating that indometacin does not prevent the effect of allopregnanolone to prolong the duration of the $GABA_AR$-mediated mIPSC (τ_w in ms, one-way ANOVA P >0.05. *Post hoc* Newman Keul's test revealed no differences for allopregnanolone with or without indometacin, P >0.05, n =7-9). **(E)** Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs acquired from a representative control neurone and from equivalent neurones after an ~2 hour incubation of the brain slice with indometacin (100 μM), ganaxolone (300 nM), or both. Note for panels A, C and D the mIPSC amplitude is normalised to that of the control averaged mIPSC to facilitate comparison of their kinetics. **(F)** Histogram illustrating that indometacin in a concentration-dependant manner (30 – 100 μM) prevents the effect of ganaxolone to prolong the $GABA_AR$-mediated mIPSC (τ_w in ms; one-way ANOVA P < 0.05. *Post hoc* Newman Keul's test revealed differences for ganaxolone 300 nM with or without indometacin 30 -100 μM, **P < 0.05, n = 8-10. Ctrl = control; Allo = allopregnanolone; Ganax = ganaxolone; DHP = dihydroxyprogesterone; Indo = indometacin

Table 23 The 3α-HSD inhibitor indometacin (indo) reduces the effect of ganaxolone (Ganax) but not allopregnanolone (Allo) incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT mice.

	WT Control (n=35)	**WT 30µM Indo (n=6)**	**WT 100µM Indo (n=7)**	**WT 100nM Allo (n=9)**	**WT 100nM Allo & 100µM Indo (n=7)**	**WT 300nM Ganax (n=10)**	**WT 300nM Ganax & 30µM Indo (n=10)**	**WT 300nM Ganax & 100µM Indo (n=8)**
Peak amp (pA)	-59 ± 2	-53 ± 2	-53 ± 2	-78 ± 2	-77 ± 4	-82 ± 6	**-63 ± 3	**-54 ± 4
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ±0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-222 ± 6	-235 ± 13.5	-932 ± 84	-915 ± 10	-749 ± 88	**-374 ± 39	**-289 ± 30
T70 (ms)	**5.2 ± 0.2**	**5.1 ± 0.2**	**5.8 ± 0.2**	**18.6 ± 1.4**	**17.0 ± 0.5**	**13.4 ± 1**	****8.7 ± 0.7**	****6.3 ± 0.3**
Tau w (ms)	**4.0 ± 0.1**	**4.6 ± 0.2**	**4.2 ± 0.1**	**12.5 ± 1**	**13.5 ± 0.8**	**10.9 ± 1**	****6.2 ± 0.5**	****5.4 ± 0.3**
Frequency (Hz)	17 ± 2	14 ± 2	14 ± 2	15 ± 3	10 ± 2	20 ± 3	23 ± 2	#9.3 ± 1

***P* < 0.05; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to 300nM ganaxolone

These results with ganaxolone were unexpected given the lack of impact that indometacin had on the effectiveness of allopregnanolone. Indeed, inhibiting 3α-HSD with provera had no impact on ganaxolone, but prevented the effect of DHP on $GABA_AR$ mIPSCs in layer 2/3 cortical neurones. This raises the question as to whether ganaxolone is an allosteric modulator of the $GABA_AR$ and a precursor to a more active neurosteroid such as allopregnanolone. Alternatively, could indometacin be a silent competitive steroid antagonist at the $GABA_AR$ and prevent the action of ganaxolone by that mechanism? (These questions are explored in the Discussion).

4.14 The effect of pipette-applied ganaxolone and indometacin on layer 2/3 cortical $GABA_A$ mIPSCs in mature WT mice (P60-75).

In order to explore whether indometacin could be a silent competitive antagonist at the $GABA_AR$ (and therefore prevent modulation by ganaxolone), recordings were made with indometacin in the pipette. Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones with indometacin ± ganaxolone presented in the recording pipette. These recordings were compared against separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 24).

The mIPSC decay (τ_W) of L2/3 cortical neurones of WT mice was unchanged by the presence of indometacin in the recording pipette and indometacin had no effect on the modulatory action of ganaxolone (τ_W: control = 4.0 ± 0.1 ms, n = 35, indometacin 100 μM = 3.9 ± 0.4 ms, n = 5; ganaxolone 10 μM = 6.1 ± 0.3 ms, n = 6; ganaxolone 10 μM & indometacin 100 μM = 7.3 ± 0.6 ms, n = 4; One-way ANOVA with *Post hoc* Newman Keul's test revealed that indometacin did not affect the baseline decay time of mIPSCs (P >0.05) and there was no difference between ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM, P >0.05; Table 24; Figure 35). The T_{70} was similarly unaffected by the presence of indometacin (Table 24). The inability of indometacin to prevent the modulation of the $GABA_AR$

mIPSCs by ganaxolone is inconsistent with the concept that indometacin could be a silent antagonist of the $GABA_AR$. Consequently, it seems more likely that indometacin may inhibit the action of ganaxolone by preventing its conversion to a more active compound (such as allopregnanlone).

Table 24 The effect of pipette-applied ganaxolone and indometacin on layer 2/3 cortical $GABA_AR$ mIPSCs in mature WT mice (P60-75).

	WT Control (n=35)	**WT 100µM Indo (n=5)**	**WT 10µM Ganax (n=6)**	**WT 10µM Ganax & 100µM Indo (n=4)**
Peak amplitude (pA)	-59 ± 2	-75 ± 2	-69 ± 6	-72 ± 7
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-306 ± 28	-420 ± 55	-494 ± 68
T70 (ms)	**5.2 ± 0.2**	**4.8 ± 0.2**	**8.1 ± 0.6**	**9.4 ± 0.5**
Tau w (ms)	**4.0 ± 0.1**	**3.9 ± 0.4**	**6.1 ± 0.3**	**7.3 ± 0.6**
Frequency (Hz)	17 ± 2	16 ± 4	16 ± 2	14 ± 2

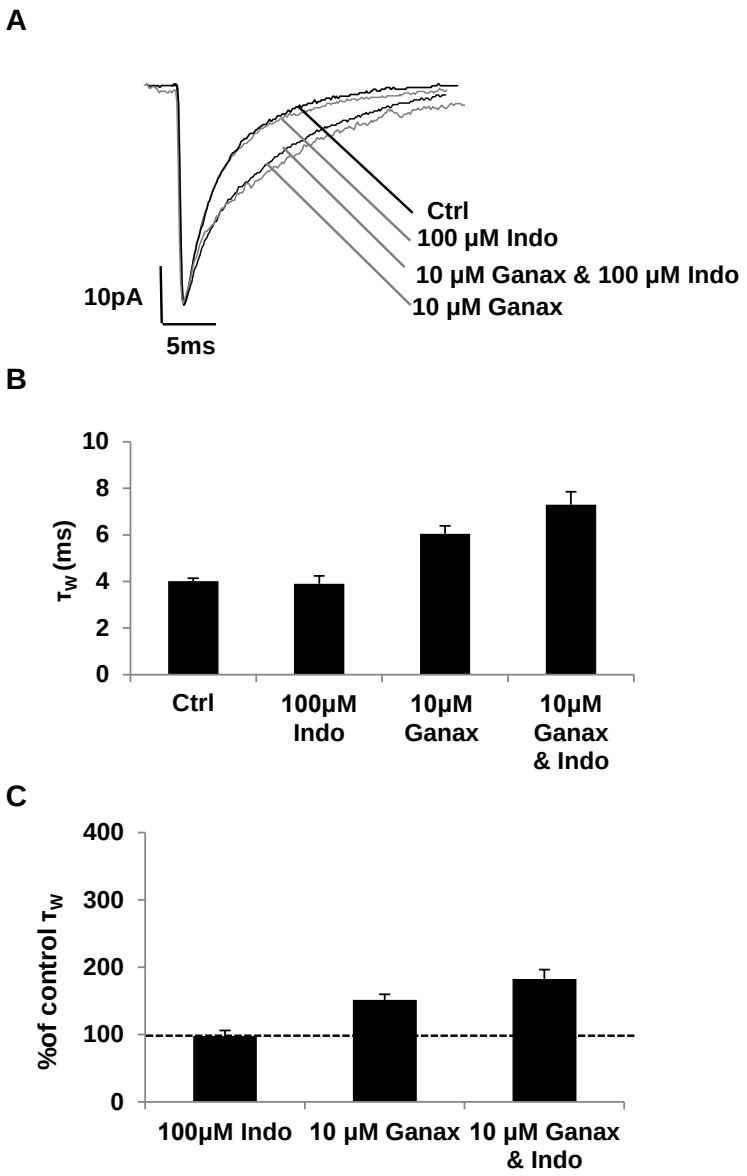

Figure 35 The 3α-HSD enzyme inhibitor indometacin does not suppress the acute effects of intracellularly applied ganaxolone on $GABA_AR$ mIPSCs of mature cortical neurones (in contrast to its inhibitory effect on ganaxolone incubation treatment).

(A) Superimposed exemplar averaged $GABA_AR$-mediated mIPSCs acquired from a representative control mature L2/3 cortical neurone and from equivalent neurones after the intracellular application of ganaxolone (10 μM), indometacin (100 μM) or both. **(B)** Histogram illustrating that intracellular indometacin made no impact on the effectiveness of intracellular ganaxolone (Student's unpaired t tests $P > 0.05$ for ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM, n = 4-6). **(C)** Histogram illustrating that intracellular indometacin made no impact on the effectiveness of intracellular ganaxolone expressed as a percentage of control (Student's unpaired t tests $P > 0.05$ for ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM, n = 4-6). Ctrl = control; Ganax = ganaxolone; Indo = indometacin

Chapter 5: The role of neurosteroids on mature layer 2/3 neurones from the cerebral cortex of *ob/ob* and *db/db* mice.

The previous Chapter characterised the neurosteroid modulation of the L2/3 cortical $GABA_A$Rs in mature WT mice. This late stage of development was chosen because it facilitated a comparison with the *ob/ob* mouse model of type-2 diabetes mellitus (T2DM). The *ob/ob* mouse develops super-morbid obesity and exhibits a neuropathic phenotype and consequently develops hypersensitivity to pain by the age of P60-75 (Drel *et al.,* 2006; Latham *et al.,* 2009). To date, there are no published reports of the electrophysiological characterisation of $GABA_A$R function for the *ob/ob* mouse. Whole-cell voltage-clamp recordings of synaptic $GABA_A$R mIPSCs from cortical L2/3 neurones were made from mature C57/BL6 wild type mice, *ob/ob* mice and also from wild type mice from the same genetic background strain as the *ob/ob* mouse B6.V-Lep^{+}/OlaHsd (aged P60-75).

The *ob/ob* mice are deficient in the hormone leptin and it was considered that leptin itself might modulate $GABA_A$R function (Solovyova *et al.,* 2009). In order to exclude such a direct effect of leptin as a cause of any changes observed, recordings were additionally made in a second mouse model of T2DM, the *db/db* mouse. As described in the Introduction section, the *db/db* mouse is able to synthesise leptin but lacks the leptin receptor, consequently developing super-morbid obesity and a more severe diabetic phenotype than the *ob/ob* mouse (Bates *et al.,* 2005; Cefalu, 2006; Neubauer & Kulkarni, 2006). The *db/db* mouse also exhibits a neuropathic phenotype at P60-75, which includes significant impairments of thermal nociception and nerve conduction velocity, consistent with sensory loss (Sullivan *et al.,* 2006).

Mice that were used for electrophysiological (or behavioural experiments) were weighed in order to confirm the presence of obesity. The *ob/ob* and *db/db* mice (P60-75) both had significantly greater body weights than the respective WT animals of the same age (C57/BL6 = 26.8 ± 0.3g, n = 32; B6.V-*Lep*$^{+}$ = 24.7 ± 0.3g, n = 49; BKS.Cg-*Dock7*^{m}*+* = 22.8 ± 1g, n = 5; *ob/ob* = 35.6 ± 0.4g, n = 50; *db/db* = 42.2 ± 0.8g, n = 9; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between both diabetic mice and all three strains of WT mice, $P < 0.05$; Figure 36). These data are consistent with reports of *ob/ob* and *db/db* body mass in the literature (Latham *et al.,* 2009; Bates *et al.,* 2005).

5.1 A comparison of synaptic $GABA_AR$ mIPSCs of cortical layer 2/3 neurones in adult WT, *ob/ob* and *db/db* mice.

Whole-cell voltage-clamp recordings were made from L2/3 pyramidal neurones of the cerebral cortex of C57/BL6 (WT) mice, *ob/ob* mice and *db/db* mice in addition to the lean WT littermates for both models of T2DM (B6.V-*Lep*$^{+}$/OlaHsd and BKS.Cg-*Dock7*^{m}*+/Dock7*^{m}+/OlaHsd respectively). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 25).

There was a modest but significant reduction in the mIPSC decay (τ_W) of cortical L2/3 pyramidal neurones between the diabetic mice and the corresponding WT littermates (*Lep*$^{+}$/OlaHsd = 3.9 ± 0.2 ms, n = 18; *ob/ob* = 3.5 ± 0.1 ms, n = 25; Student's unpaired t test, $P < 0.05$; BKS.Cg-*Dock7*^{m}*+* = 4.3 ± 0.3 ms, n = 8; *db/db* = 3.5 ± 0.1 ms, n = 18; Student's unpaired t test, $P < 0.05$; Table 25; Figure 37). The mIPSC T_{70} was also significantly different between the diabetic mice and the WT littermates (Table 25). However, there was no significant difference in the τ_W, or the $T_{70,}$ between the three WT strains, suggesting that the observed reduction of mIPSC decay time in the *ob/ob* and *db/db* mouse models was not related to inter-strain variability (see Table 25; Figure 37). These findings suggest that either the $GABA_ARs$ of the T2DM mice may be less sensitive to endogenous neurosteroids, or the levels of endogenous neurosteroid is reduced in T2DM, or both.

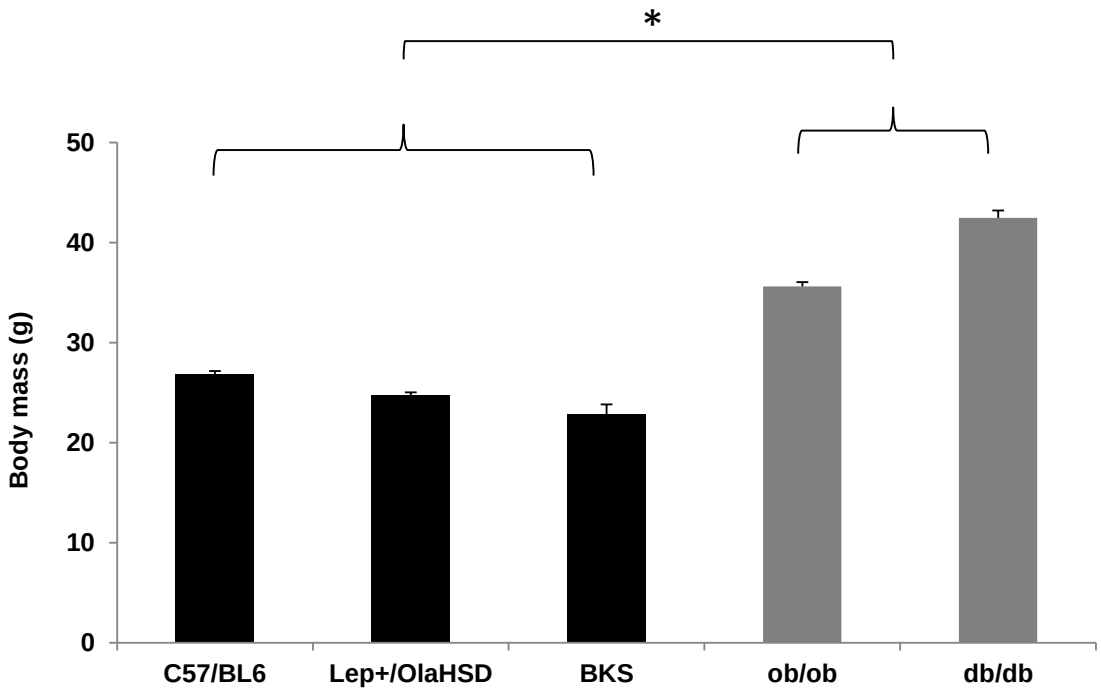

Figure 36 *Ob/ob* & *db/db* mice exhibit a morbid obesity phenotype on a normal diet, consistent with reports in the literature.

Histogram illustrating that the mature *ob/ob* and *db/db* mice used experimentally had significantly greater body weights than the respective WT animals of the same age. (C57/BL6 = 26.8 ± 0.3g, n = 32, B6.V-*Lep*$^{+}$ = 24.7 ± 0.3g, n = 49; BKS.Cg-*Dock7*^{m}*+* = 22.8 ± 1g, n = 5; *ob/ob* = 35.6 ± 0.4g, n = 50; *db/db* = 42.2 ± 0.8g, n = 9; One-way ANOVA $P < 0.05$. *Post hoc* Newman Keuls test revealed significant differences between both diabetic mice and all three strains of WT mice *$P < 0.05$).

Table 25 A comparison of synaptic $GABA_AR$ mIPSCs of cortical layer 2/3 neurones in adult WT, *ob/ob* and *db/db* mice.

	WT C57 BL6 Control (n=35)	**WT B6.V-*Lep*$^+$/ OlaHsd (n=18)**	**WT BKS.Cg-*Dock7*^{m}+ (n=8)**	***Ob/ob*** **(n=25)**	***Db/db*** **(n=18)**
Peak amp (pA)	-59 ± 2	-64 ± 2	-74 ± 5	-66 ± 2	-60 ± 2
Rise Time (ms)	0.4 ± 0.1	0.5 ±0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	-265 ±11	-333 ± 26	-255 ± 12	-223 ± 8
T70 (ms)	**5.2 ± 0.2**	**5.3 ± 0.2**	**5.6 ± 0.4**	***4.7 ± 0.1**	***4.6 ± 0.1**
Tau w (ms)	**4.0 ± 0.1**	**3.9 ± 0.2**	**4.3 ± 0.3**	***3.5 ± 0.1**	***3.5 ± 0.1**
Frequency (Hz)	17 ± 2	24 ± 2	14.8 ± 2	21 ± 3	19 ± 3

*$P < 0.05$; unpaired Student's t test, in comparison to appropriate strain-matched control (P60-75)

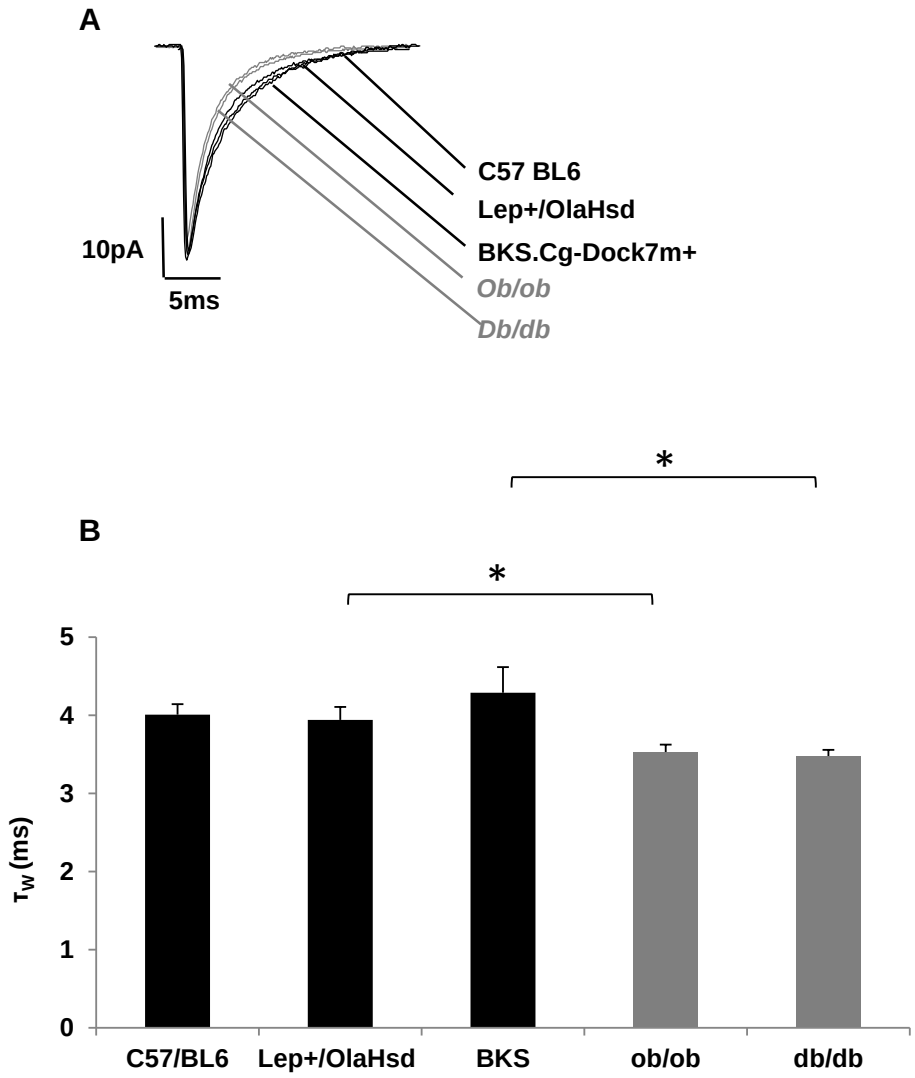

Figure 37 Mature cortical neurones of *ob/ob* & *db/db* mice exhibit shorter $GABA_AR$ mIPSCs compared to those from three different WT strains.

(A) Superimposed exemplar $GABA_ARs$ mIPSCs from a representative control mature L2/3 cortical neurone of three WT strains (black) and two diabetic phenotypes (grey). **(B)** Histogram illustrating the shorter cortical $GABA_AR$ mIPSC τ_w of the diabetic mice and their corresponding WT strains (*Lep*$^+$/OlaHsd = 3.9 ± 0.2 ms, n = 18; *ob/ob* = 3.5 ± 0.1 ms, n = 25; Student's unpaired t test **P* <0.05; BKS.Cg-*Dock7*^{m}*+* = 4.3 ± 0.3 ms, n = 8; *db/db* = 3.5 ± 0.1 ms, n = 18; Student's unpaired t test **P* <0.05).

5.2 The effect of cyclodextrin on layer 2/3 cortical $GABA_A$ mIPSCs in mature *ob/ob* mice (P60-75).

As described in the Introduction section, the cyclodextrins (CDs) are relatively large barrel-shaped molecules that may be classified according to the number of glucose residues (Brown, 2012; Shu *et al.,* 2004, 2007). The three principle types of CD are the α-CD hexamer, the β-CD heptamer and the γ-CD octomer (Cooper *et al.,* 2005; Davis & Brewster, 2004). The largest of these cyclodextrins, γ-CD appears to be the most effective for the sequestration of neurosteroids (Brown, 2012; Shu *et al.,* 2004, 2007). Whole-cell voltage-clamp recordings were made in layer 2/3 cortical pyramidal neurones with cyclodextrin present in the recording pipette (*i.e.* the optimal method of administration as described in the previous chapter). These recordings were compared to separate control recordings (*i.e.* they were not paired), where the recording pipette contained only the intracellular solution. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 26).

The mIPSC decay (τ_W) of L2/3 cortical pyramidal neurones of the *ob/ob* mouse was significantly decreased in the presence of γ-CD, but not by α-CD, or by β-CD (*ob/ob* control = 3.5 ± 0.1 ms, n = 25; *ob/ob* γ-CD = 3.0 ± 0.2 ms, n = 6; *ob/ob* α-CD = 3.7 ± 0.2 ms, n = 5; *ob/ob* β-CD = 3.6 ± 0.2 ms, n = 6; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed that the γ-CD group were significantly different from all of the other groups, $P < 0.05$; Table 26; Figure 38). The T_{70} was similarly decreased in the presence of γ-CD, but not by α-CD, or by β-CD; Table 26). These data are consistent with those described for the WT mouse in the previous chapter. These results suggest that there is a still a neurosteroid tone in the *ob/ob* mouse, but that either it is reduced in comparison to the WT, or the $GABA_ARs$ of the *ob/ob* mouse are less sensitive to neurosteroids.

Table 26 The effect of intracellular α/β/γ – cyclodextrin (CD) 0.5mM on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT and *ob/ob* mice.

	WT Control (n=35)	**WT α-CD (n=7)**	**WT β-CD (n=6)**	**WT γ-CD (n=15)**	***Ob/ob* (n=25)**	***Ob/ob* α-CD (n=5)**	***Ob/ob* β-CD (n=6)**	***Ob/ob* γ-CD (n=6)**
Peak amp (pA)	-59 ± 2	-55 ± 3	-56 ± 3	-58 ± 2	-66 ± 2	-65 ± 3	-57 ± 3	-71 ± 5
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	-243 ± 15	-261 ± 16	**-185 ± 8	-255 ± 12	-260 ± 16	-222 ± 14	-225 ± 18
T70 (ms)	**5.2 ± 0.2**	**5.7 ± 0.6**	**5.9 ± 0.5**	****3.7 ± 0.2**	**4.7 ± 0.1**	**4.8 ± 0.2**	**4.6 ± 0.2**	****3.8 ± 0.2**
Tau w (ms)	**4.0 ± 0.1**	**4.1 ± 0.5**	**4.5 ± 0.4**	****2.9 ±0.1**	**3.5 ± 0.1**	**3.7 ± 0.2**	**3.6 ± 0.2**	****3.0 ± 0.2**
Frequency (Hz)	17 ± 2	18 ± 4	18 ± 5	18 ± 2	21 ± 3	17 ± 3	20 ± 4	19 ± 3

**$P < 0.05$; one-way ANOVA (with *Post hoc* Newman Keul's test), in comparison to strain-matched control (P60-75)

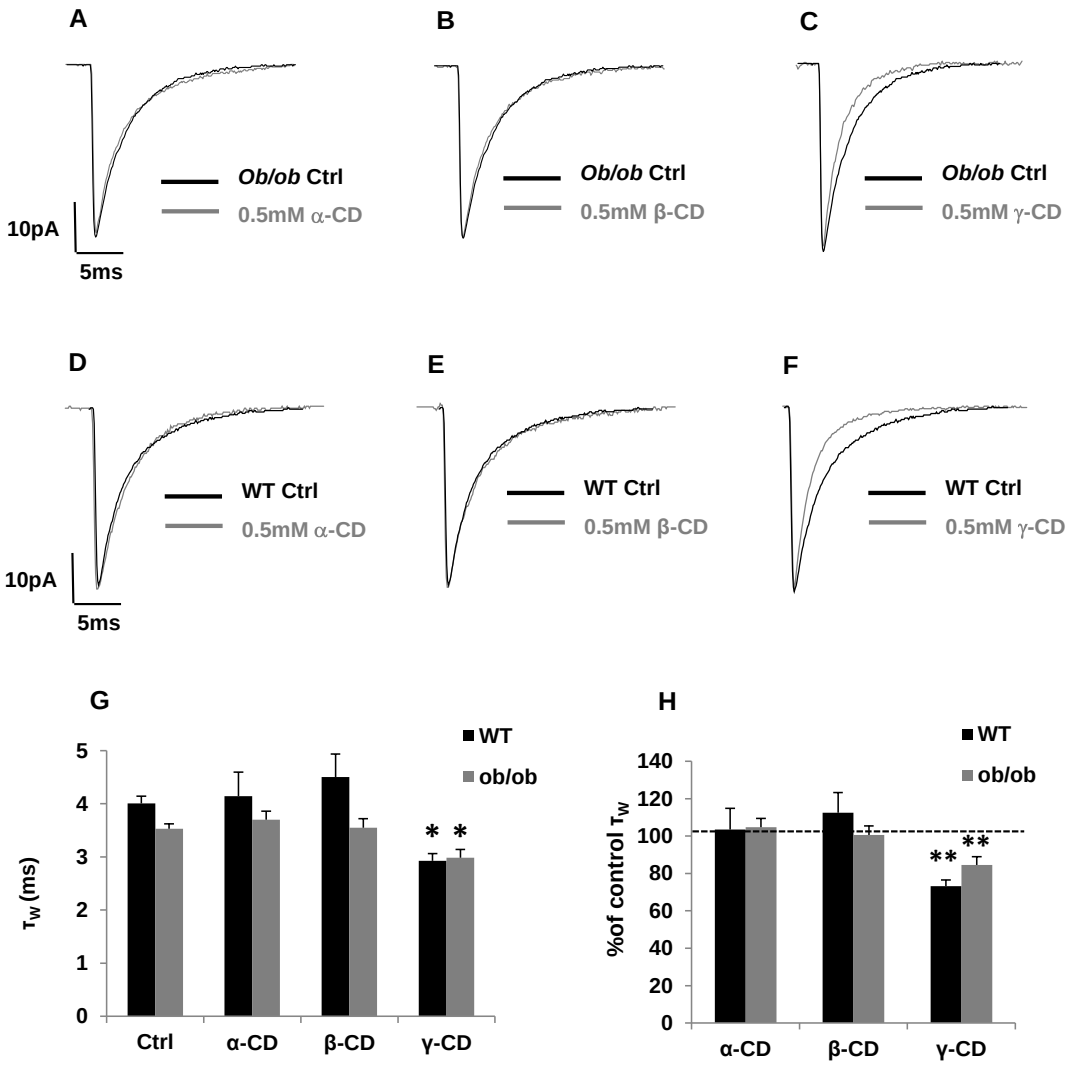

Figure 38 Intracellular γ-CD (but not α-CD or β-CD) reduces the duration of $GABA_AR$ mIPSCs of mature cortical neurones from WT and *ob/ob* mice.

(A-C) Superimposed exemplar $GABA_AR$-mediated mIPSCs from representative mature L2/3 cortical neurones of *ob/ob* mice and from equivalent neurones with 0.5 μM α-CD, β-CD or γ-CD administered intracellularly *via* the recording pipette. Note recordings of mIPSCs for both control and cyclodextrin-treated neurones did not commence for ~6 mins after establishing the whole-cell voltage-clamp to allow the compound to equilibrate (Evans & Marty, 1986). **(D-F)** Superimposed exemplar $GABA_ARs$ mIPSCs from representative mature L2/3 cortical neurones of WT mice and from equivalent neurones with 0.5 μM α-CD, β-CD or γ-CD administered intracellularly. **(G)** Histogram illustrating that intracellular γ-CD (but not α-CD or β-CD) decreases the $GABA_AR$ mIPSC τ_w of mature L2/3 cortical neurones of *ob/ob* mice (*ob/ob* Ctrl = 3.5 ± 0.1 ms, n = 25, *ob/ob* γ-CD = 3.0 ± 0.2 ms, n = 6; *ob/ob* α-CD = 3.7 ± 0.2 ms, n = 5; *ob/ob* β-CD = 3.6 ± 0.2 ms, n = 6; One-way ANOVA $P < 0.05$. *Post hoc* Newman Keul's test revealed that the γ-CD group were significantly different from all of the other groups, $^*P < 0.05$). The values for WT mice (black bars) are incorporated for comparison and to highlight that the τ_w value is the same for WT and *ob/ob* mice in the presence of γ-CD. **(H)** Histogram illustrating that intracellular γ-CD (but not α-CD or β-CD) decreases the $GABA_AR$ mIPSC τ_w of mature L2/3 cortical neurones of *ob/ob* mice (grey bars; expressed as a percentage of control) 85 ± 4%, 105 ± 5%, 101 ± 5% respectively *vs.* unpaired representative *ob/ob* Ctrl (One-way RM ANOVA $^{**}P < 0.05$). Ctrl = control. CD = cyclodextrin.

5.3 A comparison of the effects of γ-cyclodextrin on cortical mIPSCs of adult WT, *ob/ob* and *db/db* mice.

As described above, γ-CD reduced the decay time of $GABA_AR$ mIPSCs in *ob/ob* and WT mice. In order to determine that neither the strain, nor the lack of leptin (directly) was an important factor in the shortening of $GABA_AR$ mIPSC decay time, it was decided to make recordings from a second mouse model of T2DM and the appropriate strain-matched WT. Whole-cell voltage-clamp recordings were made from L2/3 neurones of the cerebral cortex of C57/BL6 (WT) mice, *ob/ob* mice and *db/db* mice, in addition to the lean WT littermates for both models of T2DM (B6.V-*Lep*$^{+}$/OlaHsd and BKS.Cg-*Dock7*^{m}*+/Dock7*^{m}+/OlaHsd respectively), with γ-CD present in the pipette. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 27).

Table 27 A comparison of the effects of 0.5mM γ-cyclodextrin (γ-CD) on cortical $GABA_AR$ mIPSCs of adult WT, *ob/ob* and *db/db* mice (P60-75).

	WT C57 BL6 Control γ-CD (n=15)	**WT B6.V-*Lep*$^{+}$/ OlaHsd γ-CD (n=11)**	**WT BKS.Cg-*Dock7*^{m}+ γ-CD (n=5)**	***Ob/ob* γ-CD (n=6)**	***Db/db* γ-CD (n=9)**
Peak amp (pA)	-58 ± 2	-55 ± 1	-58 ± 3	-71 ± 5	-54 ± 2
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-185 ± 8	-175 ± 7	-214 ± 19	-225 ± 18	-170 ± 11
T70 (ms)	**3.7 ± 0.2**	**3.8 ± 0.2**	**4.4 ± 0.2**	**3.8 ± 0.2**	**3.9 ± 0.1**
Tau w (ms)	**2.9 ± 0.1**	**3.0 ± 0.1**	**3.3 ± 0.2**	**3.0 ± 0.2**	**2.9 ± 0.1**
Frequency (Hz)	18 ± 2	*25 ± 3	12 ± 1	19 ± 3	13 ± 3

In the presence of intracellular γ-CD, there was no significant difference in the mIPSC decay (τ_W) of cortical layer 2/3 neurones between all five types of mice (C57/BL6 γ-CD = 2.9 ± 0.1 ms, n = 15; *Lep*$^+$/OlaHsd γ-CD = 3.0 ± 0.1 ms, n = 11; BKS.Cg-*Dock7*^{m}*+* γ-CD = 3.3 ± 0.2 ms, n = 5; *ob/ob* γ-CD = 3.0 ± 0.2 ms, n = 6; *db/db* γ-CD = 2.9 ± 0.2 ms, n = 9; One-way ANOVA, $P > 0.05$; Table 27; Figure 28). When the data were normalised and expressed as a percentage of their respective controls, there were no significant differences in the effect of γ-CD between all five types of mice (C57/BL6 γ-CD = 73 ± 3%, n = 15; *Lep*$^+$/OlaHsd γ-CD = 76 ± 3%, n = 11; BKS.Cg-*Dock7*^{m}*+* γ-CD = 77 ± 4%, n = 5; *ob/ob* γ-CD = 85 ± 4%, n = 6; *db/db* γ-CD = 82 ± 5%, n = 9; One-way RM ANOVA, $P > 0.05$).

Similarly, there was no significant difference in the T_{70} of $GABA_AR$-mediated mIPSCs with pipette-applied γ-CD (Table 27). These findings suggest that synaptic $GABA_AR$ function is very similar across all the types of mice included in the study when the endogenous neurosteroid tone is removed by γ-CD. The results are also consistent with the hypothesis that there is a neurosteroid tone at P60-75, but that it is reduced in both mouse models of diabetic neuropathy. However, the data do not exclude the possibility that the sensitivity of L2/3 cortical $GABA_ARs$ to neurosteroids may be reduced.

5.4 The effect of the acute application of allopregnanolone, or ganaxolone, on $GABA_AR$ mIPSCs of mature *ob/ob* mice.

In the previous chapter, the acute bath application of allopregnanolone and ganaxolone induced modest prolongations of the $GABA_AR$ mIPSC decay time in L2/3 cortical neurones of WT mice. In this section, the effect of the acute application of these neuroactive steroids is investigated in order to examine $GABA_AR$ sensitivity in the *ob/ob* mouse. Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones from *ob/ob* mice *before* and ten minutes *after* the bath application of allopregnanolone, or ganaxolone. The neuroactive steroids were bath-applied after at least four minutes of stable recording enabling the comparison of paired recordings. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 28).

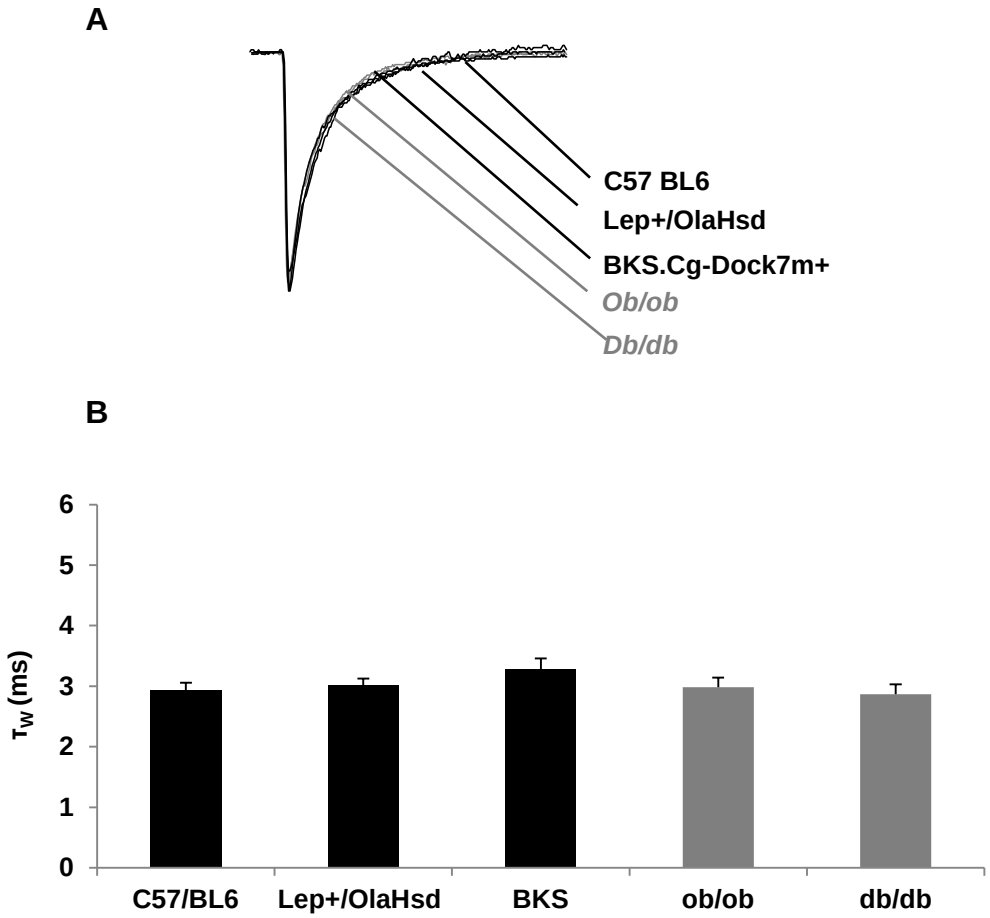

Figure 39 Intracellular γ-CD reduces the duration of $GABA_AR$ mIPSCs of mature cortical neurones from five different types of mice to a similar baseline level.

(A) Superimposed exemplar $GABA_AR$-mediated mIPSCs from a representative control mature L2/3 cortical neurone of three WT strains (black) and two diabetic phenotypes (grey) with 0.5 μM γ-CD administered intracellularly. **(B)** Histogram illustrating that there was no significant difference between all five strains of mice in the presence of intracellular γ-CD (C57/BL6 γ-CD = 2.9 ± 0.1 ms, n = 15, *Lep*$^+$/OlaHsd γ-CD = 3.0 ± 0.1 ms, n = 11; BKS.Cg-*Dock7*^{m}*+* γ-CD = 3.3 ± 0.2 ms, n = 5; *ob/ob* γ-CD = 3.0 ± 0.2 ms, n = 6; *db/db* γ-CD = 2.9 ± 0.2 ms, n = 9; One-way ANOVA $P > 0.05$). The relevant controls are presented in Figure 37.

Table 28 The acute application of allopregnanolone (Allo) or ganaxolone (Ganax) prolongs the decay time of $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT and *ob/ob* mice.

	WT Pre 1μM Allo (n=7)	**WT Post 1μM Allo (n=7)**	***Ob/ob* Pre 1μM Allo (n=7)**	***Ob/ob* Post 1μM Allo (n=7)**	**WT Pre 1μM Ganax (n=6)**	**WT Post 1μM Ganax (n=6)**	***Ob/ob* Pre 1μM Ganax (n=6)**	***Ob/ob* Post 1μM Ganax (n=6)**
Peak amp (pA)	-68 ± 3	-67 ± 5	-64 ± 2	*-71 ± 3	-57 ± 4	*-66 ± 6	-75 ± 5	-80 ± 3
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-284 ± 7	-305 ± 14	-253 ± 15	*-323 ± 18	-232 ± 15	*-309 ± 31	-325 ± 27	-372 ± 35
T70 (ms)	**5.2 ± 0.4**	***5.8 ± 0.5**	**4.9 ± 0.2**	***5.9 ± 0.3**	**5.2 ± 0.2**	***6.1 ± 0.4**	**5.1 ± 0.2**	***5.6 ± 0.3**
Tau w (ms)	**4.0 ± 0.3**	***4.5 ± 0.4**	**3.7 ± 0.1**	***4.4 ± 0.2**	**4.0 ± 0.2**	***4.7 ± 0.2**	**3.8 ± 0.2**	***4.4 ± 0.2**
Frequency (Hz)	26 ± 6	21 ± 4	11 ± 1	11 ± 1	22 ± 5	21 ± 5	16 ± 3	17 ± 1

*$P < 0.05$; paired Student's t test, in comparison to strain-matched control (P60-75)

In the *ob/ob* mouse, allopregnanolone produced a modest (18 ± 4%) prolongation of exponential decay time in the $GABA_AR$-mediated mIPSCs of layer 2/3 cortical neurones (τ_W: control = 3.7 ± 0.1 ms, n = 7; allopregnanolone 1 μM = 4.4 ± 0.2 ms, n = 7; Paired Student's t test, P <0.05). When the data for τ_W were normalised, there was no significant difference between allopregnanolone 1 μM in the WT (12 ± 3%) and the *ob/ob* mice (18 ± 4%) respectively (Two-way RM ANOVA, P >0.05; Figure 40). Similarly, allopregnanolone induced a modest prolongation of the mIPSC T_{70} (Table 28). In the *ob/ob* mouse, ganaxolone (1μM) also induced only a modest (15 ± 4%) prolongation of exponential decay time of the $GABA_AR$-mediated mIPSCs (τ_W: control = 3.8 ± 0.2 ms, n = 6; ganaxolone 1 μM = 4.4 ± 0.2 ms, n = 6; Student's paired t test, P <0.05). When the data for τ_W were normalised, there was no significant difference between ganaxolone 1 μM in the WT (19 ± 4%) and the *ob/ob* mice (15 ± 4%) respectively (Two-way RM ANOVA, P >0.05; Figure 40). The mIPSC T_{70} was also modestly prolonged by ganaxolone (Table 28). These data indicate that the acute application of allopregnanolone, or ganaxolone, has a similarly modest effect on the $GABA_AR$ mIPSCs of *ob/ob* cortical neurones to that described for WT in the previous Chapter. Furthermore, these results suggest that the $GABA_ARs$ of cortical neurones from both types of mice may exhibit comparable sensitivity to neuroactive steroids (Figure 40).

5.5 The effect of prolonged incubation of allopregnanolone, on $GABA_AR$-mediated mIPSCs of mature *ob/ob* and *db/db* mice.

As described in the previous Chapter, the pre-incubation of the brain slice preparation with allopregnanolone, or ganaxolone, produced a concentration-dependent prolongation of cortical $GABA_AR$-mediated mIPSCs of adult WT mice. To explore the sensitivity of cortical synaptic $GABA_ARs$ of *ob/ob* and *db/db* mice to allopregnanolone and ganaxolone, whole-cell voltage-clamp recordings were made in layer 2/3 cortical neurones after approximately two hours incubation with these neuroactive steroids. Such recordings were compared to those of separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, rise time, charge transfer, decay kinetics and frequency (Table 29).

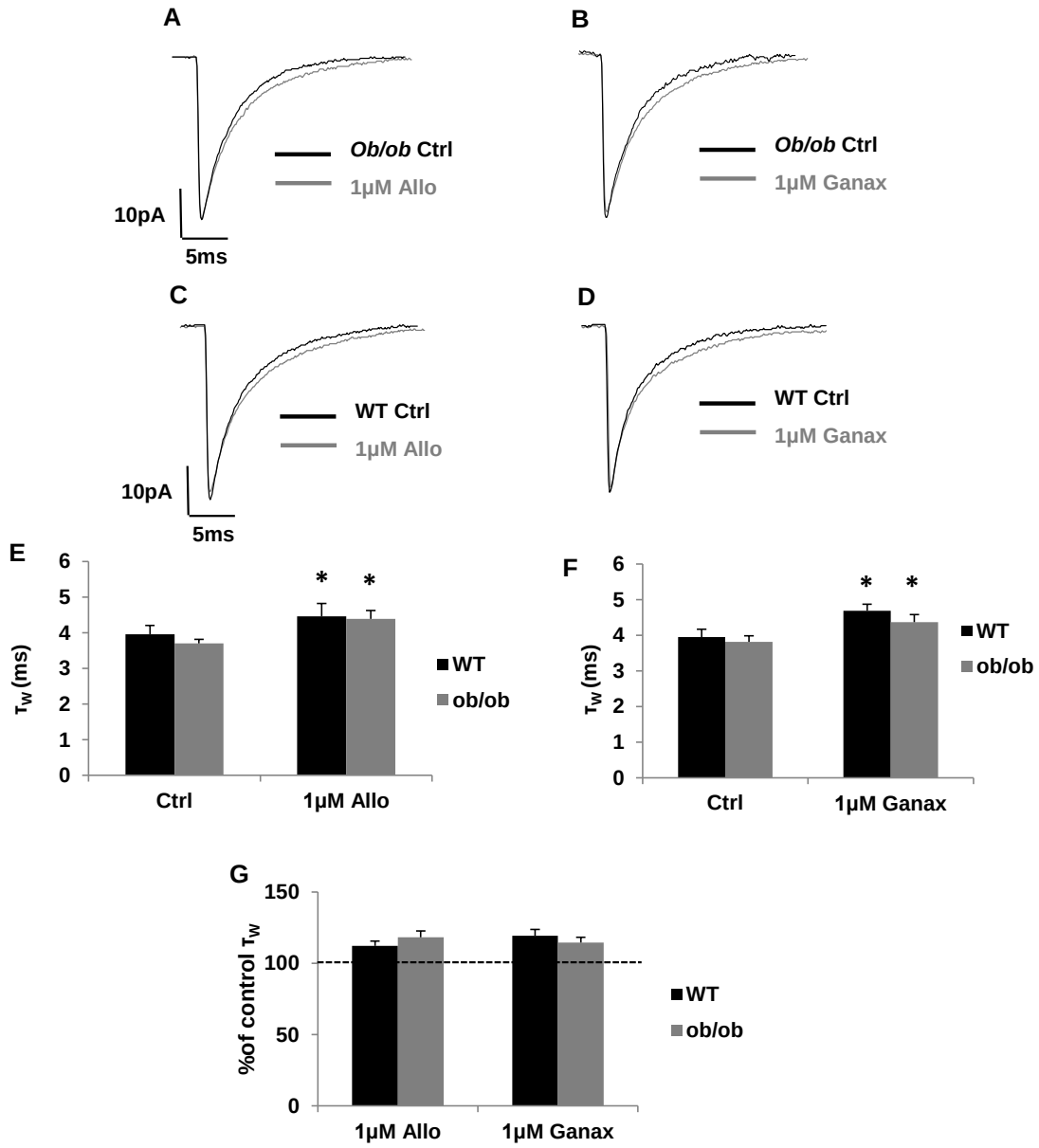

Figure 40 Acute exposure (10-20mins) of WT & ob/ob mature cortical neurones to allopregnanolone (1μM) or ganaxolone (1μM) modestly enhances the function of synaptic $GABA_A$Rs by a similar margin.

(A & **B)** Superimposed exemplar $GABA_A$R-mediated mIPSCs from cortical neurones of *ob/ob* mice before and after acute exposure to 1μM allopregnanolone, or ganaxolone. **(C** & **D)** Superimposed exemplar $GABA_A$Rs mIPSCs from cortical neurones of WT mice before and after acute exposure to 1μM allopregnanolone, or ganaxolone. **(E** & **F)** Histograms illustrating the significant effect of 1μM allopregnanolone, or ganaxolone on $GABA_A$R mIPSCs of *ob/ob* cortical neurones (grey bars; Student's paired t test **P* <0.05 for each drug treatment *vs.* its paired control recording). The values for WT cortical neurones (black bars) are included for comparison. **(G)** Histogram illustrating the effect of the neurosteroids with the data normalised relative to control recordings (*i.e.* expressed as a percentage of the control recording). When the data for τ_w was normalised, there was no significant difference between the WT and *ob/ob* mice for allopregnanolone treatment (WT = 12 ± 3%; *ob/ob* = 18 ± 4%; Two-way RM ANOVA *P* >0.05), or ganaxolone treatment (WT = 19 ± 4%; *ob/ob* = 15 ± 4%; Two-way RM ANOVA *P* >0.05). Ctrl = control; Allo = allopregnanolone; Ganax = ganaxolone.

Table 29 The effect of allopregnanolone (Allo) incubation treatment on $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT, *ob/ob* and *db/db* mice.

	WT Control (n=35)	***Ob/ob* (n=25)**	***Db/db* (n=18)**	**WT 100nM Allo (n=9)**	***Ob/ob* 100nM Allo (n=6)**	**WT 300nM Allo (n=9)**	***Ob/ob* 300nM Allo (n=8)**	***Db/db* 300nM Allo (n=6)**
Peak amp (pA)	-59 ± 2	-66 ± 2	-60 ± 2	**-78 ± 2	**-81 ± 2	**-87 ± 5	**-94 ± 4	*-99 ± 3
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-255 ± 12	-223 ± 8	**-932 ± 84	**-987 ± 82	**-1489 ± 136	**-1540 ± 100	*-1604 ± 81
T70 (ms)	**5.2 ± 0.2**	**4.7 ± 0.1**	**4.6 ± 0.1**	****18.6 ± 1**	****18.7 ± 1**	****27 ± 1**	****25.8 ± 0.9**	***25.4 ± 0.6**
Tau w (ms)	**4.0 ± 0.1**	**3.5 ± 0.1**	**3.5 ± 0.1**	****12.5 ± 1**	****12.8 ± 1**	****19 ± 1**	****18.8 ± 1**	***18.6 ± 0.6**
Frequency (Hz)	17 ± 2	21 ± 3	19 ± 3	15 ± 3	**10 ± 1	**11 ± 1	**11 ± 2	13 ± 2

***P* < 0.05; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to strain-matched control (P60-75)

A two-hour incubation of the *ob/ob* brain slice preparation with allopregnanolone produced a clear concentration-dependent prolongation of the duration of cortical $GABA_AR$-mediated mIPSCs (τ_W: control = 3.5 ± 0.1 ms, n = 25; allopregnanolone 100 nM = 12.8 ± 1 ms, n = 6; allopregnanolone 300 nM = 18.8 ± 1 ms, n = 8; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed significant differences between control and both concentrations of allopregnanolone, which increased τ_W to 362 ± 27% and 534 ± 28% of control respectively (P <0.05; Figure 41). Similarly, the mIPSC T_{70} was significantly prolonged by allopregnanolone (Table 29).

A two-hour incubation of the *db/db* brain slice with allopregnanolone (300nM) had a similar effect to that of the *ob/ob* neurones on the duration of the cortical $GABA_AR$-mediated mIPSCs (τ_W: control = 3.5 ± 0.1 ms, n = 18; allopregnanolone 300 nM = 18.6 ± 0.6 ms, n = 6; Student's t test, P >0.05; Table 29; Figure 41). Similarly, the mIPSC T_{70} was significantly prolonged by allopregnanolone (Table 29). These results reveal that allopregnanolone has a similar effect on L2/3 cortical $GABA_AR$ mIPSC decay time of WT, *ob/ob* and *db/db* mice (τ_W: C57/BL6 WT allopregnanolone 300 nM = 19.0 ± 1ms, n = 9; *ob/ob* allopregnanolone 300 nM = 18.8 ± 1 ms, n = 8; *db/db* allopregnanolone 300 nM = 18.6 ± 0.6 ms, n = 6; One-way ANOVA, P > 0.05; Table 29; Figure 41). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 29) are distinctly shorter, the data for τ_W were normalised, but there was no significant difference in the effect of between the three types of mice. Allopregnanolone 300 nM increased τ_W to similar percentages of the strain representative controls (C57/BL6 WT = 480 ± 22%, n = 9; *ob/ob* = 534 ± 28%, n = 8; *db/db* = 534 ± 18%, n = 6; One-way RM ANOVA, P > 0.05; Figure 41; Figure 50). These results suggest that the sensitivity of cortical $GABA_ARs$ in diabetic mice is similar to aged-matched WT mice.

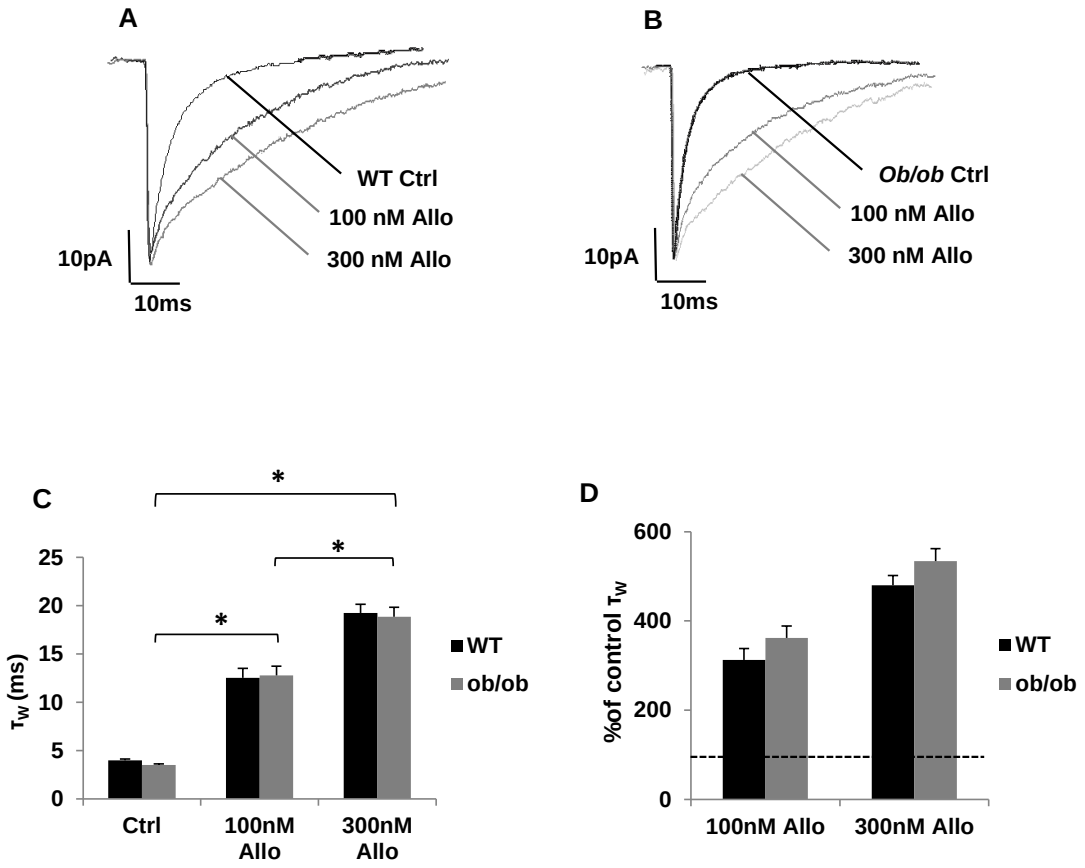

Figure 41 Prolonged exposure (~2 hrs) of WT & *ob/ob* mature cortical neurones to allopregnanolone (100-300 nM) greatly enhances the function of synaptic $GABA_A$Rs by a similar margin.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from representative control cortical neurones from mature WT mice and from equivalent neurones after ~2 hour brain slice incubation with 100 nM and 300 nM allopregnanolone. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from representative control cortical neurones from mature *ob/ob* mice and from equivalent neurones after ~2 hour brain slice incubation with 100 nM and 300 nM allopregnanolone. **(C)** Histogram illustrating the concentration-dependent effect of allopregnanolone on the cortical $GABA_A$R mIPSCs of *ob/ob* mice (grey; *ob/ob* control = 3.5 ± 0.1 ms, n = 25; allopregnanolone 100 nM = 12.8 ± 1 ms, n = 6; allopregnanolone 300 nM = 18.8 ± 1 ms, n = 8; One-way ANOVA P <0.05. *Post hoc* Newman Keuls test revealed significant differences between control and both concentrations of allopregnanolone, which increased τ_w to 362 ± 27% and 534 ± 28% of control respectively *P <0.05). Note that there is no significant difference in response between the two types of mice (WT 300 nM Allo = 19.0 ± 1 ms, n = 9; *ob/ob* 300 nM Allo =18.8 ± 1 ms, n = 8; One-way RM ANOVA P > 0.05). **(D)** Histogram comparing the concentration-dependent effects of allopregnanolone on the duration of $GABA_A$Rs mIPSC τ_w expressed as a percentage of control for WT (black) and *ob/ob* (grey) mice. The histogram illustrates that there is no significant difference in the effect of 300 nM allopregnanolone on the cortical $GABA_A$Rs mIPSCs of WT and *ob/ob* neurones (C57/BL6 WT = 480 ± 22%, n = 9; *ob/ob* = 534 ± 28%, n = 8; One-way RM ANOVA P > 0.05). Ctrl = control; Allo = allopregnanolone.

5.6 The effect of ganaxolone incubation on $GABA_AR$ mIPSCs in mature *ob/ob* and *db/db* mice.

A two-hour incubation of the *ob/ob* brain slice preparation with ganaxolone produced a clear concentration-dependent increase of the duration of cortical $GABA_AR$ mIPSCs. Interestingly, the prolongation was comparatively greater than for equivalent WT neurones (τ_W: control = 3.5 ± 0.1 ms, n = 25; ganaxolone 30 nM = 7.2 ± 0.5 ms, n = 11; ganaxolone 100 nM = 8.4 ± 0.7 ms, n = 10; ganaxolone 300 nM = 15.4 ± 1 ms, n = 9; ganaxolone 1 μM = 20.6 ± 1 ms, n = 10 One-way ANOVA, P <0.05; Table 30; Table 31; Figure 42). When the values were expressed as a percentage of control, there were significant differences between all the concentrations of ganaxolone, τ_W was increased to 204 ± 13%, 237 ± 20%, 437 ± 42% and 584 ± 29% of control respectively, One-way RM ANOVA, with *post hoc* Newman Keul's test, P <0.05; Figure 42). Similarly, the mIPSC T_{70} was significantly prolonged by ganaxolone (Table 30; Table 31).

In the *db/db* layer 2/3 cortical neurones, $GABA_AR$ mIPSCs were prolonged by a similar extent to that observed in the *ob/ob* mouse (τ_W: control = 3.5 ± 0.1 ms, n = 18; ganaxolone 300 nM = 14.8 ± 0.4 ms, n = 4; ganaxolone 1 μM = 22.0 ± 1.6 ms, n = 8; One-way ANOVA, P < 0.05; *post hoc* Newman Keul's test revealed significant differences between all three treatments, P <0.05; Table 30; Table 31). The mIPSC T_{70} was also significantly prolonged by ganaxolone (Table 30; Table 31). These results reveal that a two-hour pre-incubation with ganaxolone caused a more pronounced prolongation of cortical mIPSCs of *ob/ob* and *db/db c.f.* WT (τ_W: WT ganaxolone 300 nM = 10.9 ± 1 ms, n = 10; *ob/ob* ganaxolone 300 nM = 15.4 ± 1 ms, n = 9; *db/db* ganaxolone 300 nM = 14.8 ± 0.4 ms, n = 4; One-way ANOVA, P < 0.05; *post hoc* Newman Keul's test revealed significant differences between all three strains, P < 0.05; Table 31). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 31) are distinct, the data for τ_W were normalised, and there were significant difference in the effect of between the three types of mice.

Table 30 The effect of ganaxolone (Ganax) incubation treatment (30-100nM) on $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT, *ob/ob* and *db/db* mice

	WT Control (n=35)	***Ob/ob* (n=25)**	***Db/db* (n=18)**	**WT 30nM Ganax (n=10)**	***Ob/ob* 30nM Ganax (n=11)**	**WT 100nM Ganax (n=10)**	***Ob/ob* 100nM Ganax (n=10)**
Peak amp (pA)	-59 ± 2	-66 ± 2	-60 ± 2	**-61 ± 3	-60 ± 4	**-66 ± 4	**-74 ± 4
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-255 ± 12	-223 ± 8	**-300 ± 26	-399 ± 35	**-440 ± 39	**-573 ± 66
T70 (ms)	**5.2 ± 0.2**	**4.7 ± 0.1**	**4.6 ± 0.1**	****6.3 ± 0.4**	****9.3 ± 0.5**	****9.6 ± 0.6**	****11.6 ± 0.9**
Tau w (ms)	**4.0 ± 0.1**	**3.5 ± 0.1**	**3.5 ± 0.1**	****5.1 ± 0.3**	****7.2 ± 0.5**	****7.2 ± 0.5**	****8.4 ± 0.7**
Frequency (Hz)	17 ± 2	21 ± 3	19 ± 3	**11 ± 1	13 ± 2	15 ± 3	17 ± 4

***P* < 0.05; One-way ANOVA, (with *Post hoc* Newman Keul's test), in comparison to strain-matched control (P60-75)

Table 31 The effect of ganaxolone (Ganax) incubation treatment (300nM, 1μM or 3μM) on $GABA_AR$ mIPSCs of L2/3 cortical neurones (P60-75) of WT, *ob/ob* and *db/db* mice

	WT Control (n=35)	***Ob/ob* (n=25)**	***Db/db* (n=18)**	**WT 300nM Ganax (n=10)**	***Ob/ob* 300nM Ganax (n=9)**	***Db/db* 300nM Ganax (n=4)**	**WT 1μM Ganax (n=10)**	***Ob/ob* 1μM Ganax (n=10)**	***Db/db* 1μM Ganax (n=8)**	**WT 3μM Ganax (n=8)**
Peak amp (pA)	-59 ± 2	-66 ± 2	-60 ± 2	**-82 ± 6	**-80 ± 7	**-79 ± 8	**-97 ± 9	**-126 ± 5	**-117 ± 3	**-153 ± 6
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Charge transfer (fC)	-242 ± 9	-255 ± 12	-223 ± 8	**-749 ± 88	**-1104 ± 178	**-1004 ± 81	**-1575 ± 195	**-2267 ± 162	**-2193 ± 83	**-4396 ± 268
T70 (ms)	**5.2 ± 0.2**	**4.7 ± 0.1**	**4.6 ± 0.1**	****13.4 ± 1**	****20.7 ± 2**	****19.3 ± 0.7**	****24.2 ± 1**	****27.5 ± 1**	****28.5 ± 1.4**	****44.2 ± 2**
Tau w (ms)	**4.0 ± 0.1**	**3.5 ± 0.1**	**3.5 ± 0.1**	****10.9 ± 1**	****15.4 ± 1**	****14.8 ± 0.4**	****18.8 ± 1**	****20.6 ± 1**	****22 ± 1.6**	****32.5 ± 1**
Frequency (Hz)	17 ± 2	21 ± 3	19 ± 3	20 ± 3	13 ± 2	10 ± 2	13.5 ± 2	12 ± 1	10 ± 2	**8 ± 1

***P* < 0.05; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to strain-matched control (P60-75)

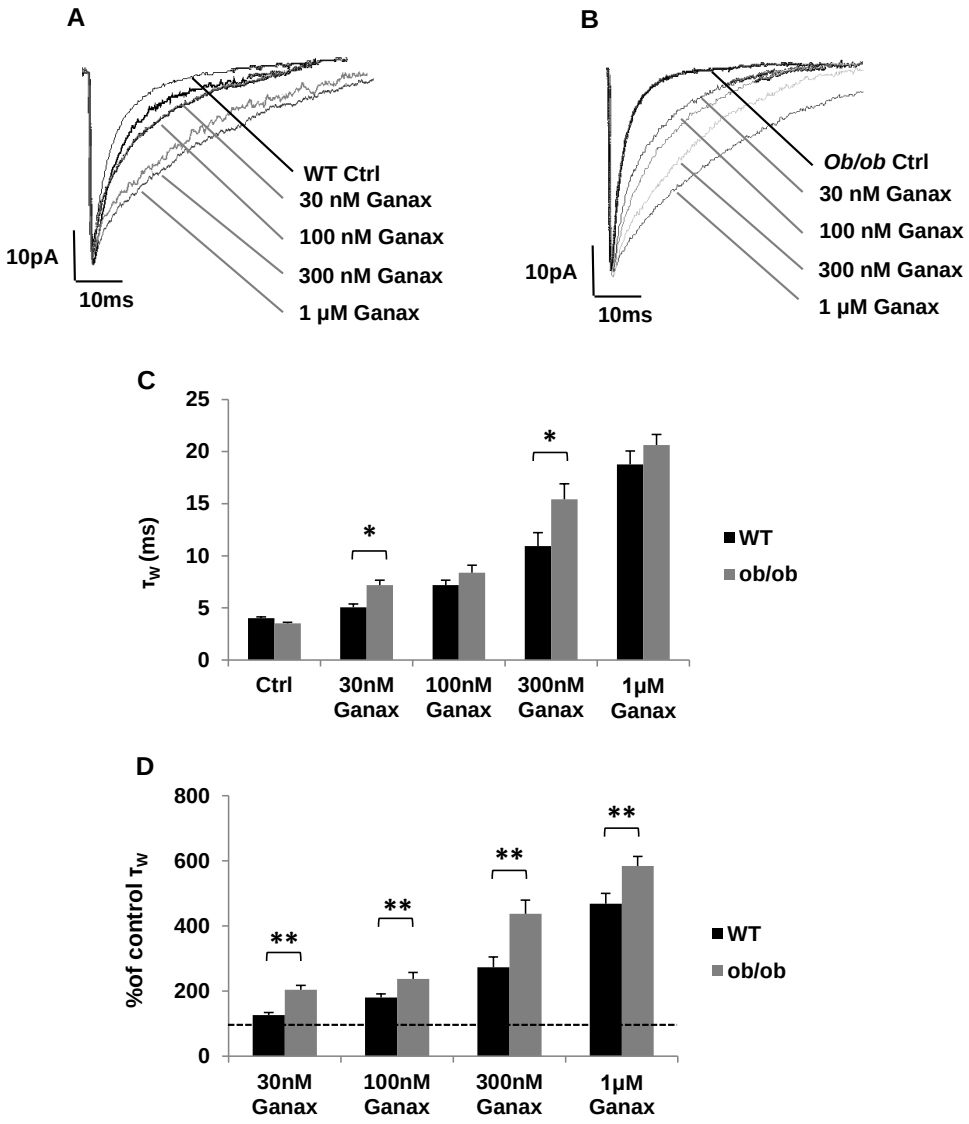

Figure 42 Prolonged exposure (~2 hrs) of mature cortical neurones to ganaxolone (30 nM- 1 μM) greatly enhances the function of synaptic $GABA_A$Rs in a concentration-dependent manner and has an exaggerated effect in *ob/ob* mice compared to WT mice.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from representative control cortical neurones from mature WT mice and from equivalent neurones after ~2 hour brain slice incubation with 30 nM - 1 μM ganaxolone. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from representative control cortical neurones from mature *ob/ob* mice and from equivalent neurones after ~2 hour brain slice incubation with 30 nM - 1 μM ganaxolone. **(C)** Histogram illustrating the concentration-dependent effect of ganaxolone 30 nM - 1 μM on the cortical $GABA_A$Rs mIPSCs of *ob/ob* mice (grey bars; One-way ANOVA P <0.05). Note the exaggerated effect of ganaxolone incubation treatment on the cortical $GABA_A$Rs mIPSCs of *ob/ob* mice (grey bars) in comparison to WT mice (black bars) for 30 nM and 300 nM ganaxolone (One-way RM ANOVA, $^*P$ < 0.05 respectively). **(D)** Histogram comparing the concentration-dependent effects of ganaxolone on the duration of $GABA_A$Rs mIPSC τ_w expressed as a percentage of control for WT (black) and *ob/ob* (grey) mice. The histogram illustrates that ganaxolone has an exaggerated effect in *ob/ob* mice (WT 30 nM = 126 ± 8%, *ob/ob* = 204 ± 13%, $^{**}P$ < 0.05; WT 100 nM = 180 ± 11%, *ob/ob* 100 nM = 237 ± 20%, $^{**}P$ < 0.05; WT 300 nM = 273 ± 32%, *ob/ob* 300 nM= 437 ± 42%, $^{**}P$ < 0.05; WT 1 μM = 468 ± 32%; *ob/ob* 1 μM= 584 ± 29%; One-way RM ANOVA $^{**}P$ < 0.05). Ctrl = control; Ganax = ganaxolone.

Ganaxolone 300 nM increased τ_W to greater percentages of the strain representative controls in the diabetic mice in comparison to the WT mice (C57/BL6 WT = 273 ± 32%, n = 10; *ob/ob* = 437 ± 42%, n = 9; *db/db* = 426 ± 13%, n = 4; One-way RM ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between the diabetic mice and the WT, $P < 0.05$, but there was no significant difference between the *ob/ob* and *db/db* mice, $P > 0.05$; Figure 50). These observations contrast with that of allopregnanolone, which caused a similar effect in all genotypes, even after normalising for different baseline values. These findings suggest that there is a difference in the effect of ganaxolone incubation treatment (but not allopregnanolone) in the *ob/ob* and *db/db* mice compared to the WT mice.

5.7 The effect of intracellular allopregnanolone, or ganaxolone, on layer 2/3 cortical $GABA_AR$ mIPSCs of mature *ob/ob* mice.

For both the WT and *ob/ob* mice acutely applied allopregnanolone and ganaxolone had modest effects on $GABA_AR$ mIPSCs exponential decay time, but were far more effective in this respect when the cortical slices were pre-incubated with the steroid for more than two hours. Pre-incubation with ganaxolone had a comparatively greater effect on cortical $GABA_AR$-mediated mIPSCs derived from the *ob/ob* than from WT mice, whereas allopregnanolone had a similar effect in both types of mice. Whole-cell voltage-clamp recordings were made in *ob/ob* L2/3 cortical neurones with allopregnanolone, or ganaxolone, present in the recording pipette. These recordings were compared against separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 32).

The mIPSC decay (τ_W) of L2/3 cortical neurones of *ob/ob* mice was increased by the presence of allopregnanolone in the recording pipette (*ob/ob* control = 3.5 ± 0.1 ms, n = 25, *ob/ob* 3 μM allopregnanolone = 5.7 ± 0.4 ms, n = 7; *ob/ob* 10 μM allopregnanolone = 15.5 ± 2 ms, n = 5; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between all groups, $P < 0.05$; Table 32,

Figure 43). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 32) are distinct, the data for τ_W were normalised for comparison. Intracellular allopregnanolone 3 - 10 μM increased τ_W to similar percentages of the strain representative controls (C57/BL6 WT 3 μM = 145 ± 19%, n = 6; *ob/ob* 3 μM = 161 ± 11%, n = 7; Two-way RM ANOVA, $P > 0.05$; C57/BL6 WT 10 μM = 403 ± 34%, n = 7; *ob/ob* 10 μM = 439 ± 60%, n = 5; Two-way RM ANOVA, $P > 0.05$; Figure 43). The T_{70} was similarly increased in the presence of allopregnanolone (Table 32). These findings are consistent with the recordings described in the previous chapter and with the hypothesis that there is no difference in the sensitivity of the cortical $GABA_AR$ to neurosteroids between the *ob/ob* and WT mice.

The mIPSC decay (τ_W) of L2/3 cortical neurones of *ob/ob* mice was increased by the presence of ganaxolone in the recording pipette (*ob/ob* control = 3.5 ± 0.1 ms, n = 25; *ob/ob* 3 μM ganaxolone = 5.0 ± 0.3 ms, n = 6; *ob/ob* 10 μM ganaxolone = 6.4 ± 0.6 ms, n = 6; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed no significant difference between the two concentrations of ganaxolone, $P > 0.05$; Table 33, Figure 44). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 33) are distinct, the data for τ_W were normalised for comparison. Intracellular ganaxolone 3 - 10 μM increased τ_W to similar percentages of the strain representative controls (C57/BL6 WT 3 μM = 138 ± 9%, n = 6; *ob/ob* 3 μM = 142 ± 7%, n = 6; One-way RM ANOVA, $P > 0.05$; C57/BL6 WT 10 μM = 151 ± 8%, n = 6; *ob/ob* 10 μM = 182 ± 18%, n = 6; One-way RM ANOVA, $P > 0.05$; Figure 44). The T_{70} was similarly increased in the presence of ganaxolone (Table 33). Here, ganaxolone did not have a concentration-dependent effect on *ob/ob* cortical $GABA_AR$ mIPSCs when acutely delivered intracellularly and the magnitude of the effect was less than that produced by equivalent pipette concentrations of allopregnanolone (Table 33; Figure 44). Note there was no difference in the $GABA_AR$-mediated mIPSC decay time between the *ob/ob* and the WT mouse when treated with intracellular ganaxolone. This finding is consistent with the recordings described above with allopregnanolone, although intracellular ganaxolone had a less pronounced effect *c.f.* allopregnanolone on $GABA_AR$ mIPSCs.

Table 32 The effect of intracellular allopregnanolone (Allo) on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT or *ob/ob* mice.

	WT Control (n=35)	**WT 3µM Allo (n=6)**	**WT 10µM Allo (n=7)**	***Ob/ob* (n=25)**	***Ob/ob* 3µM Allo (n=7)**	***Ob/ob* 10µM Allo (n=5)**
Peak amp (pA)	-59 ± 2	-64 ± 3	**-95 ± 4	-66 ± 2	-61 ± 2	**-79 ± 6
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-354 ± 49	**-1389 ± 139	-255 ± 12	-342 ± 27	**-1140 ± 173
T70 (ms)	**5.2 ± 0.2**	**7.7 ± 1**	**22.3 ± 1.4**	**4.7 ± 0.1**	**7.4 ± 0.5**	**23.0 ± 2**
Tau w (ms)	**4.0 ± 0.1**	**5.8 ± 0.8**	**16.1 ± 1.3**	**3.5 ± 0.1**	**5.7 ± 0.4**	**15.5 ± 2**
Frequency (Hz)	17 ± 2	22 ± 4	20 ± 3	16 ± 3	20 ± 4	14 ± 5

**$P < 0.05$; One-way ANOVA, in comparison to strain-matched control

Table 33 The effect of intracellular ganaxolone (Ganax) on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT or *ob/ob* mice.

	WT Control (n=35)	**WT 3µM Ganax (n=6)**	**WT 10µM Ganax (n=6)**	***Ob/ob* (n=25)**	***Ob/ob* 3µM Ganax (n=6)**	***Ob/ob* 10µM Ganax (n=6)**
Peak amp (pA)	-59 ± 2	-63 ± 4	-69 ± 6	-66 ± 2	-60 ± 2	-66 ± 4
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	**-347 ± 15	**-420 ± 55	-255 ± 12	-302 ± 21	**-401 ± 50
T70 (ms)	**5.2 ± 0.2**	****7.1 ± 0.4**	****8.1 ± 0.6**	**4.7 ± 0.1**	****6.9 ± 0.3**	****8.2 ± 0.7**
Tau w (ms)	**4.0 ± 0.1**	****5.5 ± 0.4**	****6.1 ± 0.3**	**3.5 ± 0.1**	****5.0 ± 0.3**	****6.4 ± 0.6**
Frequency (Hz)	17 ± 2	18 ± 4	16 ± 2	16 ± 3	12 ± 3	12 ± 1

***P* < 0.05; One-way ANOVA, in comparison to strain-matched control.

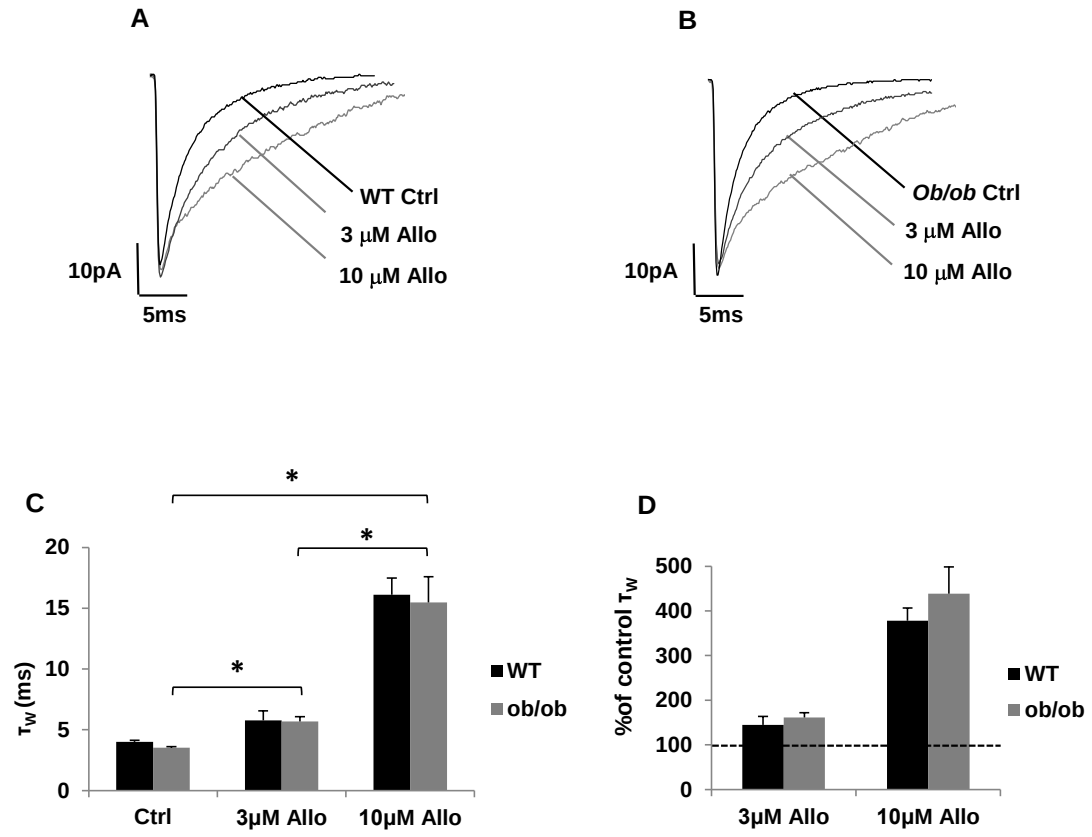

Figure 43 The intracellular application of allopregnanolone to mature cortical neurones enhances synaptic $GABA_AR$ function by a similar margin in WT and *ob/ob* mice.

(A & **B)** Superimposed exemplar $GABA_AR$-mediated mIPSCs from a representative control WT and *ob/ob* cortical neurones and from equivalent neurones with 3 - 10 μM allopregnanolone administered intracellularly. **(C)** Histogram illustrating the concentration-dependent effect of the intracellular application of allopregnanolone on the duration of $GABA_ARs$ mIPSC τ_w in *ob/ob* cortical neurones (grey bars; *ob/ob* control = 3.5 ± 0.1 ms, n = 25, *ob/ob* 3 μM allopregnanolone = 5.7 ± 0.4 ms, n = 7; *ob/ob* 10 μM allopregnanolone = 15.5 ± 2 ms, n = 5; One-way ANOVA **P* < 0.05). **(D)** Histogram illustrating the concentration-dependent effect of allopregnanolone on the duration of $GABA_ARs$ mIPSC τ_w of WT (black) and *ob/ob* cortical neurones (grey) expressed as a percentage of control. There was no significant difference in the effect of allopregnanolone on WT *vs. ob/ob* cortical $GABA_ARs$ mIPSCs (C57/BL6 WT 3 μM = 145 ± 19%, n = 6; *ob/ob* 3 μM = 161 ± 11%, n = 7; *P* > 0.05; C57/BL6 WT 10 μM = 403 ± 34%, n = 7; *ob/ob* 10 μM = 439 ± 60%, n = 5; One-way RM ANOVA *P* > 0.05). Ctrl = control; Allo = allopregnanolone.

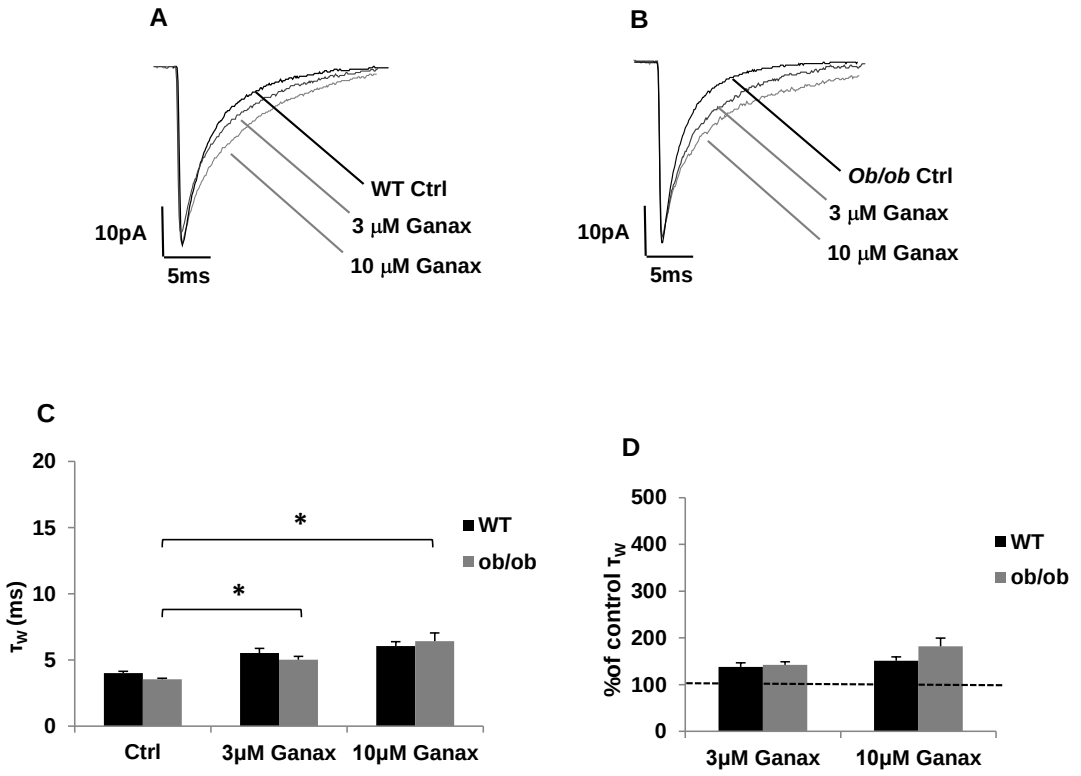

Figure 44 The intracellular application of ganaxolone to mature cortical neurones enhances synaptic $GABA_AR$ function by a similar margin in WT and *ob/ob* mice.

(A & **B)** Superimposed exemplar $GABA_ARs$ mIPSCs from a representative control WT and *ob/ob* cortical neurones and from equivalent neurones with 3 - 10 μM ganaxolone administered intracellularly. **(C)** Histogram illustrating the concentration-dependent effect of the intracellular application of ganaxolone on the duration of $GABA_ARs$ mIPSC τ_w in *ob/ob* cortical neurones (grey bars; *ob/ob* control = 3.5 ± 0.1 ms, n = 25, *ob/ob* 3 μM ganaxolone = 5.0 ± 0.3 ms, n = 6; *ob/ob* 10 μM ganaxolone = 6.4 ± .6 ms, n = 6; One-way ANOVA $^*P < 0.05$). **(D)** Histogram illustrating the concentration-dependent effect of ganaxolone on the duration of $GABA_ARs$ mIPSC τ_w of WT (black) and *ob/ob* cortical neurones (grey) expressed as a percentage of control. There was no significant difference in the effect of ganaxolone on WT *vs. ob/ob* cortical $GABA_ARs$ mIPSCs (C57/BL6 WT 3 μM = 138 ± 9%, n = 6; *ob/ob* 3 μM = 142 ± 7%, n = 6; $P > 0.05$; C57/BL6 WT 10 μM = 151 ± 8%, n = 6; *ob/ob* 10 μM = 182 ± 18%, n = 6; One-way RM ANOVA $P > 0.05$). Ctrl = control; Ganax = ganaxolone.

5.8 The effect of pipette-applied ganaxolone and indometacin on layer 2/3 cortical $GABA_A$ mIPSCs in mature *ob/ob* and WT mice (P60-75).

In the previous chapter, pipette-applied indometacin had no impact on cortical $GABA_AR$ mIPSCs in WT mice and did not alter the modulatory effect of pipette-applied ganaxolone. In order to explore whether indometacin may have a different effect on the cortical $GABA_AR$ mIPSCs of diabetic mice the experiments were repeated for the *ob/ob* mice. Whole-cell voltage-clamp recordings were made in *ob/ob* L2/3 cortical neurones with indometacin ± ganaxolone presented in the recording pipette. These recordings were compared against separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 34).

The mIPSC decay (τ_W) of L2/3 cortical neurones of *ob/ob* mice was unaffected by the presence of indometacin in the recording pipette and indometacin had no effect on the modulatory action of ganaxolone (*ob/ob* control = 3.5 ± 0.1 ms, n = 25; *ob/ob* 100 μM indometacin = 3.7 ± 0.3 ms, n = 5; *ob/ob* 10 μM ganaxolone = 6.4 ± 0.6 ms, n = 6; *ob/ob* 10 μM ganaxolone & 100 μM indometacin = 6.9 ± 0.4 ms, n = 4; One-way ANOVA. *Post hoc* Newman Keul's test revealed that indometacin did not affect the baseline decay time of mIPSCs (P >0.05) and there was no difference between ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM, P >0.05; Table 34; Figure 45). The T_{70} was similarly unchanged in the presence of indometacin (Table 34). The inability of indometacin to prevent the modulation of the $GABA_AR$ mIPSCs by ganaxolone is inconsistent with the concept that indometacin could be a silent antagonist of the $GABA_AR$ in *ob/ob* cortical neurones.

Table 34 The effect of pipette-applied ganaxolone and indometacin on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT or *ob/ob* mice.

	WT Control (n=35)	**WT 100µM Indo (n=5)**	**WT 10µM Ganax (n=6)**	**WT 10µM Ganax &100µM Indo (n=4)**	***Ob/ob* (n=25)**	***Ob/ob* 100µM Indo (n=5)**	***Ob/ob* 10µM Ganax (n=6)**	***Ob/ob* 10µM Ganax &100µM Indo (n=4)**
Peak amp (pA)	-59 ± 2	-75 ± 2	-69 ± 6	-72 ± 7	-66 ± 2	-75 ± 2	-66 ± 4	-72 ± 5
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	-306 ± 28	-420 ± 55	-494 ± 68	-255 ± 12	-228 ± 22	-401 ± 50	-497 ± 39
T70 (ms)	**5.2 ± 0.2**	**4.8 ± 0.2**	**8.1 ± 0.6**	**9.4 ± 0.5**	**4.7 ± 0.1**	**4.9 ± 0.3**	**8.2 ± 0.7**	**9.5 ± 0.3**
Tau w (ms)	**4.0 ± 0.1**	**3.9 ± 0.4**	**6.1 ± 0.3**	**7.3 ± 0.6**	**3.5 ± 0.1**	**3.7 ± 0.3**	**6.4 ± 0.6**	**6.9 ± 0.4**
Frequency (Hz)	17 ± 2	16 ± 4	16 ± 2	14 ± 2	16 ± 3	19 ± 5	12 ± 1	14 ± 1

Table 35 The effect of progesterone (Prog) incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT, *ob/ob* or *db/db* mice.

	WT Control (n=35)	**WT 1μM Prog (n=6)**	**WT 3μM Prog (n=8)**	**WT 10μM Prog (n=9)**	**WT 50μM Prog (n=9)**	***Ob/ob* (n=25)**	***Ob/ob* 1μM Prog (n=5)**	***Ob/ob* 3μM Prog (n=10)**	***Ob/ob* 10μM Prog (n=7)**	***Ob/ob* 50μM Prog (n=12)**	***db/db* 50μM Prog (n = 6)**
Peak amp (pA)	-59 ± 2	-65 ± 2	-64 ± 3	**-70 ± 2	**-91 ± 5	-66 ± 2	-68 ± 5	-78 ± 3	-79 ± 3	**-84 ± 4	-79 ± 4
Rise Time (ms)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Charge transfer	-242 ± 9	**-319 ± 19	**-339 ± 26	**-373 ± 20	**-515 ± 34	-255 ± 12	**-352 ± 34	**-409 ± 24	**-474 ± 31	**-499 ± 24	-446 ± 24
T70 (ms)	**5.2 ± 0.2**	****6.5 ± 0.5**	****6.8 ± 0.2**	****7.3 ± 0.4**	****7.6 ± 0.2**	**4.7 ± 0.1**	****6.8 ± 0.7**	****6.9 ± 0.2**	****8.4 ± 0.6**	****8.1 ± 0.4**	**7.8 ± 0.3**
Tau w (ms)	**4.0 ± 0.1**	****4.7 ± 0.3**	****5.2 ± 0.2**	****5.1 ± 0.3**	****5.3 ± 0.2**	**3.5 ± 0.1**	****5.1 ± 0.3**	****5.1 ± 0.1**	****6.1 ± 0.4**	****5.9 ± 0.3**	**5.5 ± 0.2**
Frequency (Hz)	17 ± 2	16 ± 2	17 ± 2	19 ± 2	**31 ± 3	21 ± 3	1 ± 2	**27 ± 3	17 ± 4	**29 ± 3	25 ± 4

**$P < 0.05$; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to strain-matched control (P60-75)

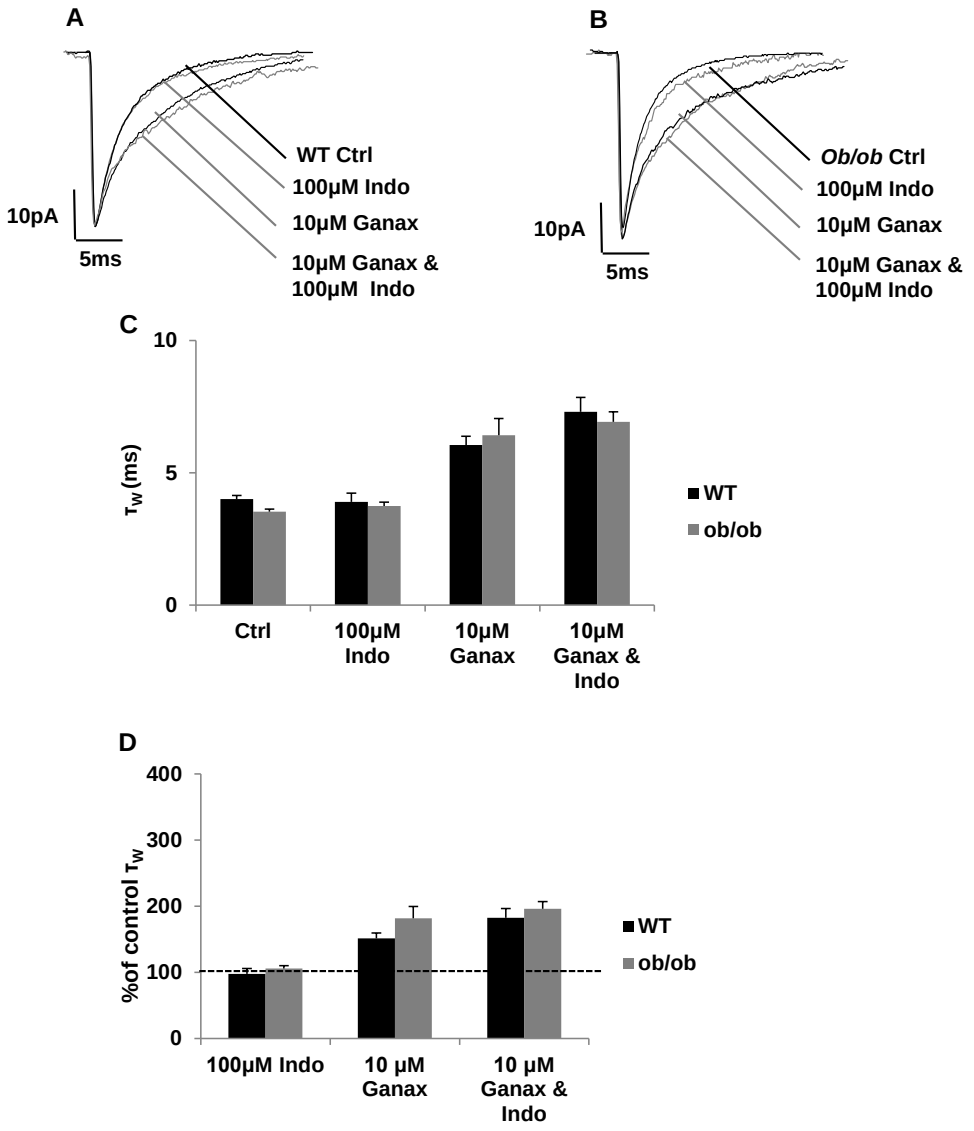

Figure 45 The 3α-HSD enzyme inhibitor indometacin does not suppress the acute effects of intracellularly applied ganaxolone on $GABA_AR$ mIPSCs of mature cortical neurones of WT or *ob/ob* mice (in contrast to its inhibitory effect on ganaxolone incubation treatment).

(A) Superimposed exemplar $GABA_AR$-mediated mIPSCs from a representative control mature WT cortical neurone and from equivalent neurones after the intracellular application of 10 μM ganaxolone, 100 μM indometacin or both. **(B)** Superimposed exemplar $GABA_ARs$ mIPSCs from a representative control mature *ob/ob* cortical neurone and from equivalent neurones after the intracellular application of 10 μM ganaxolone, 100 μM indometacin or both. **(C)** Histogram illustrating that intracellular indometacin made no impact on the modulatory action of ganaxolone (*ob/ob* Ctrl = 3.5 ± 0.1 ms, n = 25, *ob/ob* 100 μM indometacin = 3.7 ± 0.3 ms, n = 5; *ob/ob* 10 μM ganaxolone = 6.4 ± 0.6 ms, n = 6; *ob/ob* 10 μM ganaxolone & 100 μM indometacin = 6.9 ± 0.4 ms, n = 4, one-way ANOVA with *post hoc* Newman Keul's test revealed that indometacin did not affect the baseline decay time of mIPSCs (P >0.05) and there was no difference between ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM, P >0.05). **(D)** Histogram illustrating that intracellular indometacin made no impact on the effectiveness of intracellular ganaxolone expressed as a percentage of control (One-way RM ANOVA, P > 0.05 for ganaxolone 10 μM *vs*. ganaxolone 10 μM with indometacin 100 μM). Ctrl = control; Ganax = ganaxolone; Indo = indometacin.

5.9 The effect of prolonged pre-incubation with indometacin on $GABA_AR$ mIPSCs of mature *ob/ob* mice.

In addition to the experiments with indometacin in the recording pipette (above) two hour incubation with indometacin had no effect on $GABA_AR$ mIPSC decay time in layer 2/3 cortical neurones of *ob/ob* mice (τ_W: control = 3.5 ± 0.1 ms, n = 25; indometacin 100 μM = 3.8 ± 0.1 ms, n = 8; unpaired Student's t test, P <0.05). There was also no effect on the mIPSC T_{70} following two hours of incubation with indometacin. The lack of effect of indometacin in the *ob/ob* is consistent with data obtained in the WT and the idea that indometacin does not modulate the $GABA_AR$.

5.10 Can mature layer 2/3 cortical neurones from diabetic mice synthesise neurosteroids?

In the previous chapter I established that the enzymatic function (5α-R and 3α-HSD) of mature cortex of WT mice is intact and that they are able to synthesise neurosteroids when their precursors (progesterone, or DHP) are incubated with the brain slice preparation. The modulatory effects of both progesterone and DHP on $GABA_AR$ mIPSCs could be prevented by enzymatic inhibition, consistent with these neurosteroids being precursors for allopregnanolone (Belelli & Herd, 2003; Belelli & Lambert, 2005; Brown, 2012; Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003; Figure 3). In this chapter I have established that cortical $GABA_ARs$ of *ob/ob* mice are sensitive to neurosteroids and in this section I will explore the neurosteroid synthesis pathway in these diabetic mice. Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones of diabetic mice after at least two hours of pre-incubation with the neurosteroid precursors. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 35).

5.11 The effect of progesterone pre-incubation on cortical mIPSCs of mature *ob/ob* mice.

Two hours of brain slice incubation with progesterone produced a relatively modest prolongation of $GABA_AR$-mediated mIPSC decay time in the *ob/ob* mouse (τ_W: control = 3.5 ± 0.1 ms, n = 25; progesterone 1 μM = 5.1 ± 0.3 ms, n = 5; progesterone 3 μM = 5.1 ± 0.1 ms, n = 10; progesterone 10 μM = 6.1 ± 0.4 ms, n = 7; progesterone 50 μM = 5.9 ± 0.3 ms, n = 12; One-way ANOVA, P <0.05; Table 35; Figure 46). Similarly, the mIPSC T_{70} was modestly prolonged by the steroid (Table 35). In *db/db* L2/3 cortical neurones, two hours of pre-incubation with progesterone (50 μM) produced a similar effect to that observed in the *ob/ob* and the WT (τ_W: WT progesterone 50μM = 5.3 ± 0.2ms, n = 9; *ob/ob* progesterone 50μM = 5.9 ± 0.3ms, n = 12; *db/db* progesterone 50μM = 5.5 ± 0.2ms, n = 6; One-way ANOVA, P > 0.05; Table 35; Figure 46). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 35) are distinct, the data for τ_W were normalised. When the effects of 10 μM and 50 μM progesterone were expressed as a percentage of the representative control, the steroid had a greater impact on the diabetic mice (C57/BL6 WT = 134 ± 4%, n = 9; *ob/ob* = 166 ± 7%, n = 12; *db/db* = 159 ± 6%, n = 6; One-way RM ANOVA, P < 0.05; *post hoc* Newman Keul's test revealed significant differences between the WT and both types of diabetic mice, P < 0.05, but there was no intergroup difference between the *ob/ob* and *db/db* mice, P > 0.05; Figure 46; Figure 50).

These results suggest that mature L2/3 cortical neurones of *ob/ob* and *db/db* mice are able to synthesise $GABA_AR$-modulatory neurosteroids when their precursor (progesterone) is pre-incubated with the brain slice preparation. Despite the shorter control decay time of $GABA_AR$ mIPSCs in the *ob/ob* and *db/db* cortical neurones *c.f.* WT, progesterone treatment prolonged the duration of the mIPSCs to an absolute value similar to that produced by the steroid in equivalent WT neurones. This observation suggests that not only is enzymatic function (5α-R and 3α-HSD) intact in *ob/ob* and *db/db* mice, but that it may in fact be up-regulated.

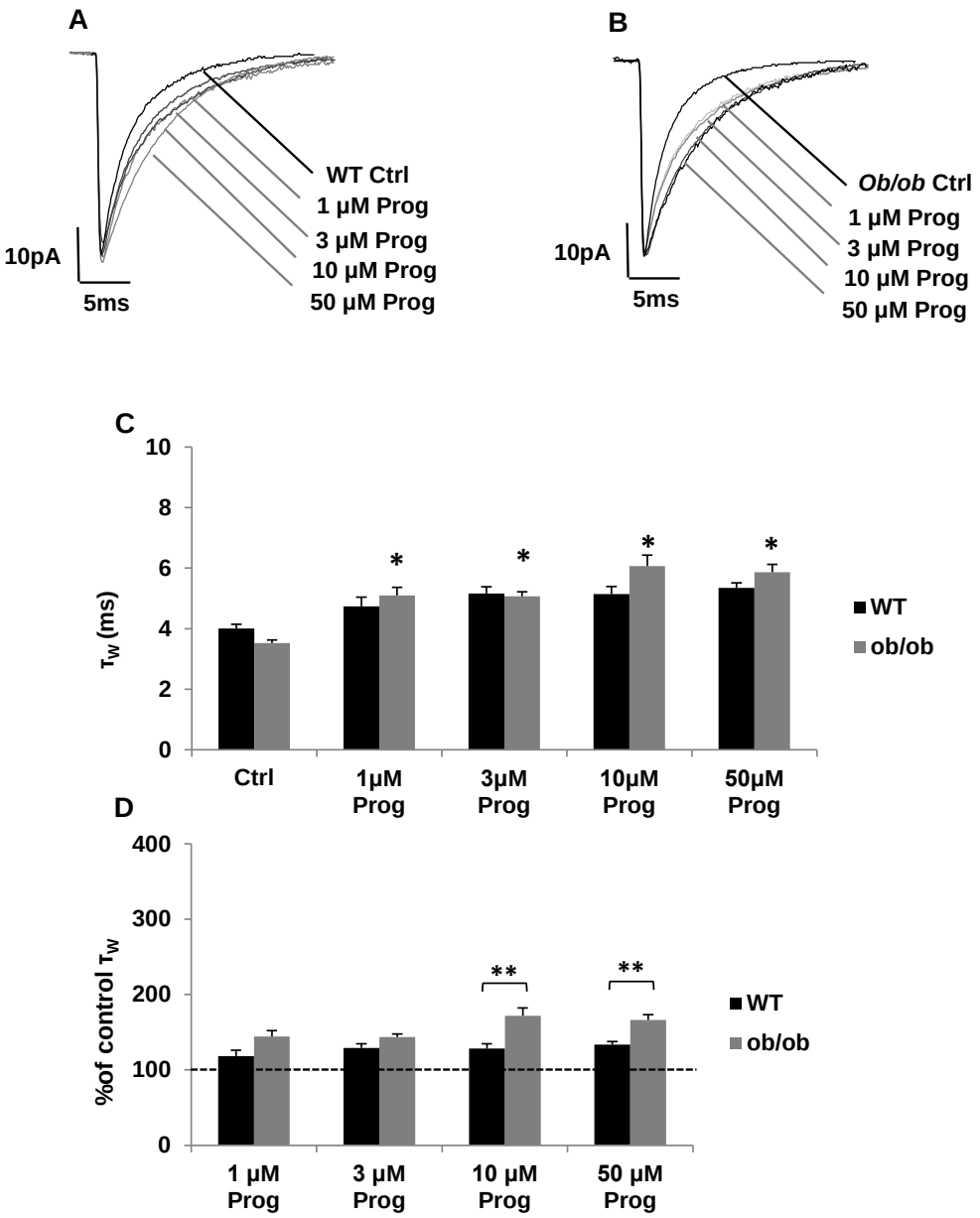

Figure 46 Prolonged exposure (~2 hrs) of mature cortical neurones to progesterone (1-50 μM) enhances the function of synaptic $GABA_A$Rs, suggesting that both WT & *ob/ob* neurones can synthesise allopregnanolone.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from a representative control mature WT cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 1 μM - 50 μM progesterone. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from a representative control mature *ob/ob* cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 1 μM - 50 μM progesterone. **(C)** Histogram illustrating the significant but modest effect of progesterone incubation treatment on *ob/ob* cortical $GABA_A$Rs mIPSCs (grey) that was not concentration-dependent (One-way ANOVA **P* <0.05). **(D)** Histogram illustrating the concentration-independent effect of progesterone on the duration of *ob/ob* $GABA_A$Rs mIPSC τ_w expressed as a percentage of control. Note that when the effect of progesterone 50 μM was expressed as a percentage of the representative control, the steroid had a greater impact on the *ob/ob* mice (C57/BL6 WT 10 μM = 129 ± 6%, n = 10, *ob/ob* 10 μM = 172 ± 10%, n = 7, $P < 0.05$; C57/BL6 WT 50 μM = 134 ± 4%, n = 9, *ob/ob* 50 μM = 166 ± 7%, n = 12, One-way RM ANOVA ***P* < 0.05). Ctrl = control; Prog = progesterone.

5.12 The influence of finasteride on the effects of progesterone on cortical mIPSCs of mature *ob/ob* mice.

In the previous Chapter I demonstrated that finasteride inhibited the effect of progesterone on the duration of cortical mIPSCs of WT mice. Whole-cell voltage-clamp recordings were made from L2/3 cortical neurones of *ob/ob* mice pre-incubated for two hours with both finasteride and progesterone. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 36). Finasteride alone had no effect on cortical mIPSC decay time of *ob/ob* mice, but it did prevent the effect of progesterone (τ_W: control = 3.5 ± 0.1 ms, n = 25; finasteride 50 μM = 3.7 ± 0.2 ms, n = 6; progesterone 50 μM = 5.9 ± 0.3 ms, n = 12; finasteride 50 μM & progesterone 50 μM = 3.4 ± 0.2 ms, n = 6; One-way ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed that progesterone 50 μM treatment was different from all the other groups, $P < 0.05$, but that there were no other intergroup differences, $P > 0.05$; Table 36; Figure 47). Similarly, the mIPSC T_{70} was unaffected by finasteride, but finasteride prevented the effect of progesterone (Table 36). These results confirm that in mature L2/3 cortical neurones of *ob/ob* (and WT mice), progesterone requires to be metabolised by 5α-R in order for it to induce prolongation of $GABA_AR$ mIPSCs.

5.13 The effect of DHP on $GABA_AR$ mIPSCs of mature *ob/ob* and *db/db* mice.

As discussed above, the progesterone metabolite DHP is the precursor of allopregnanolone, (Figure 3), but this steroid does not modulate the $GABA_AR$ directly (Belelli & Herd, 2003; Brown, 2012). Therefore, in order to modulate $GABA_AR$ mIPSCs, it requires the activity of the enzyme 3α-HSD (Belelli & Lambert, 2005; Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003). In the previous chapter I demonstrated that 3α-HSD activity is preserved in mature WT cortical neurones. Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones of *ob/ob* mice after two hours of incubation with DHP to investigate 3α-HSD function in these diabetic mice. Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency (Table 37).

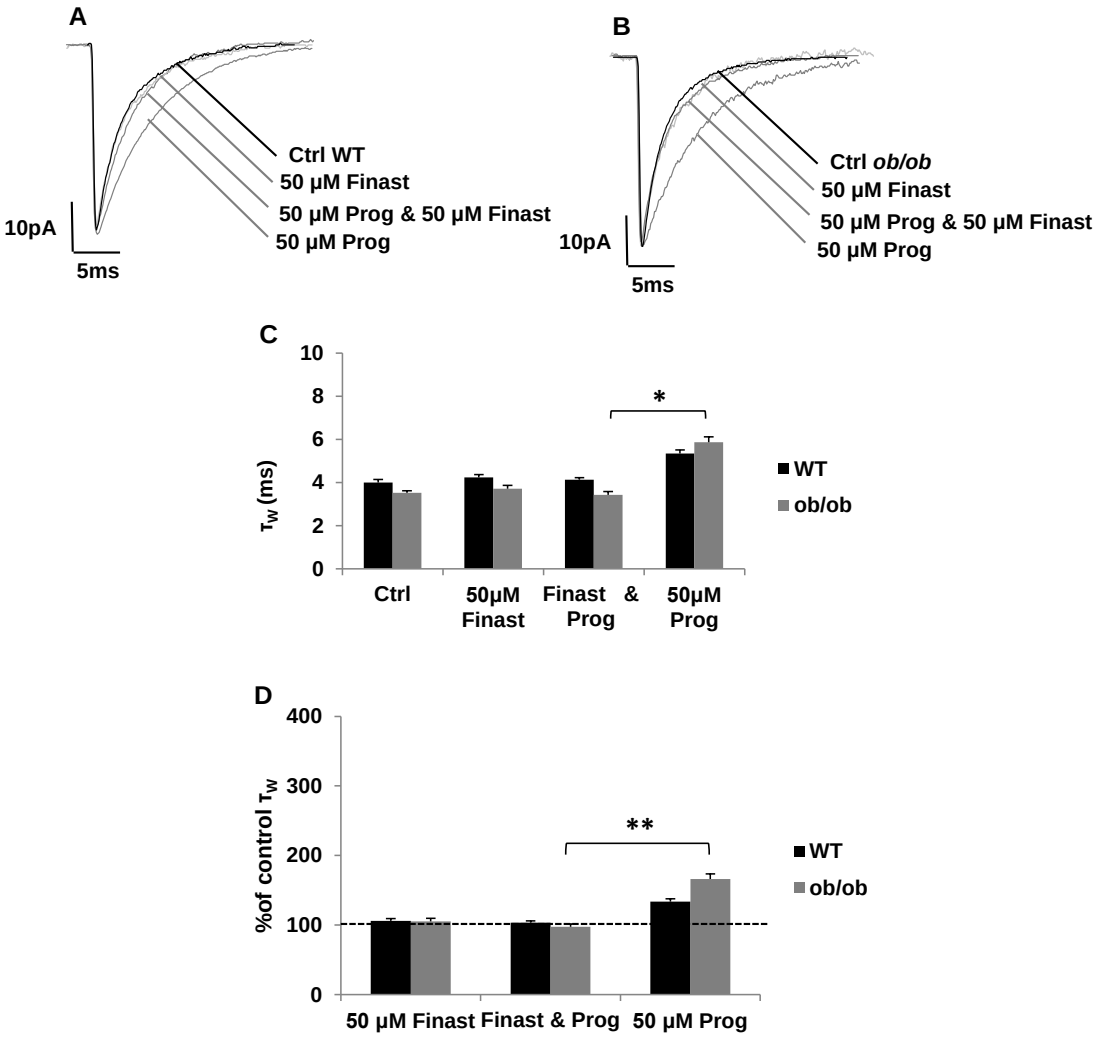

Figure 47 The 5 α-reductase enzyme inhibitor finasteride prevents the effects of progesterone incubation treatment on synaptic $GABA_A$Rs of mature cortical neurones of WT & *ob/ob* mice.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from a representative control mature WT cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 50 µM finasteride, 50 µM progesterone, or both. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from a representative control mature *ob/ob* cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 50 µM finasteride, 50 µM progesterone, or both. **(C)** Histogram illustrating the ability of finasteride to prevent the effect of progesterone incubation treatment on *ob/ob* cortical $GABA_A$Rs mIPSC τ_w (grey bars; One-way ANOVA P <0.05. *Post hoc* Newman Keul's test revealed that progesterone 50 µM treatment was different from all the other groups, $^*P < 0.05$, but that there were no other intergroup differences, $P > 0.05$). **(D)** Histogram illustrating the ability of finasteride to prevent the effect of progesterone incubation treatment on *ob/ob* cortical $GABA_A$Rs mIPSC τw (grey bars) expressed as a percentage of control (finasteride = 105 ± 4%, n = 6; finasteride & progesterone = 97 ± 4%, n= 6; progesterone = 166 ± 7%, n = 12; One-way RM ANOVA $^{**}P$ <0.05). Ctrl = control; Prog = progesterone; Finast = finasteride.

Table 36 Finasteride (Finast) prevents the effect of progesterone (Prog) incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT, *ob/ob* or *db/db* mice.

	WT Control (n=35)	**WT 50µM Finast (n=7)**	**WT 50µM Prog (n=9)**	**WT 50µM Prog & 50µM Finast (n=7)**	***Ob/ob* (n=25)**	***Ob/ob* 50µM Prog (n=12)**	***Ob/ob* 50µM Finast (n=6)**	***Ob/ob* 50µM Prog & 50µM Finast (n=6)**
Peak amp (pA)	-59 ± 2	**-67 ± 3	**-91 ± 5	-64 ± 3	-66 ± 2	**-84 ± 4	-58 ± 3	-65 ± 3
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Charge transfer (fC)	-242 ± 9	**-299 ± 19	**-515 ± 34	-269 ± 10	-255 ± 12	**-499 ± 24	-233 ± 18	-232 ± 16
T70 (ms)	**5.2 ± 0.2**	**5.7 ± 0.2**	****7.6 ± 0.2**	**5.7 ± 0.1**	**4.7 ± 0.1**	****8.1 ± 0.4**	**5.0 ± 0.2**	**4.9 ± 0.2**
Tau w (ms)	**4.0 ± 0.1**	**4.2 ± 0.1**	****5.3 ± 0.2**	**4.1 ± 0.1**	**3.5 ± 0.1**	****5.9 ± 0.3**	**3.7 ± 0.2**	**3.4 ± 0.2**
Frequency (Hz)	17 ± 2	**23 ± 2	**31 ± 3	19 ± 1.5	21 ± 3	*29 ± 3	13 ± 3	12 ± 2

***P* < 0.05; One-way ANOVA (with *Post hoc* Newman Keul's test), in comparison to strain-matched control

Table 37 The effect of DHP incubation treatment on $GABA_AR$ mIPSCs of layer 2/3 cortical neurones (P60-75) of WT, *ob/ob* and *db/db* mice.

	WT Control (n=35)	**WT 1μM DHP (n=14)**	**WT 3μM DHP (n=9)**	***Ob/ob* (n=25)**	***Ob/ob* 1μM DHP (n=18)**	***Ob/ob* 3μM DHP (n=10)**	***Db/db* (n=18)**	***Db/db* 3μM DHP (n=8)**
Peak amp (pA)	-59 ± 2	**-73 ± 4	**-69 ± 5	-66 ± 2	**-75 ± 3	-70 ± 3	-60 ± 2	*-83 ± 3
Rise Time (ms)	0.4 ± 0.1	0.4 ± 0.1	0.5 ±0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Charge transfer (fC)	-242 ± 9	**-433 ± 31	**-646 ± 33	-255 ± 12	**-792 ± 63	**-825 ± 62	-223 ± 8	*-1034 ± 57
T70 (ms)	**5.2 ± 0.2**	****8 ± 0.4**	****13.9 ± 1**	**4.7 ± 0.1**	****15.6 ± 1**	****17.6 ± 0.8**	**4.6 ± 0.1**	***19.1 ± 0.6**
Tau w (ms)	**4.0 ± 0.1**	****5.9 ± 0.2**	****10.2 ± 1**	**3.5 ± 0.1**	****10.9 ± 0.6**	****12.3 ± 0.7**	**3.5 ± 0.1**	***13.4 ± 0.6**
Frequency (Hz)	17 ± 2	16 ± 2	19 ± 6	21 ± 3	13 ± 1	14 ± 0.6	19 ± 3	16 ± 2

**$P < 0.05$; One-way ANOVA (with *Post hoc* Newman Keul's test) in comparison to strain-matched control (P60-75)

Two hours of brain slice pre-incubation with DHP (1 – 3 μM) produced a significant, concentration-dependent prolongation of layer 2/3 cortical $GABA_AR$-mediated mIPSC decay time in *ob/ob* mice (τ_W: control = 3.5 ± 0.1 ms, n = 25; DHP 1 μM = 10.9 ± 0.6 ms, n = 18; DHP 3 μM = 12.3 ± 0.7 ms, n = 10; One-way ANOVA, $P < 0.05$; Table 37; Figure 48). Similarly, the mIPSC T_{70} was significantly prolonged by DHP (Table 37). Two hours pre-incubation with 3μM DHP produced a similarly pronounced effect in L2/3 cortical neurones of the *db/db* mouse compared to the WT (τ_W: WT DHP 3 μM = 10.2 ± 0.1 ms, n = 9; *ob/ob* DHP 3 μM = 12.3 ± 0.7 ms, n = 10; *db/db* DHP 3 μM = 13.4 ± 0.6 ms, n = 8; One-way ANOVA, P <0.05; *post hoc* Newman Keul's analysis revealed intergroup differences between the WT and the diabetic mice, P <0.05; Table 37). In view of the fact that the control mIPSC decay times for the WT and diabetic mice (Table 37) are distinct, the data for τ_W were normalised. When the effect of DHP 3 μM was expressed as a percentage of the representative control, the steroid had a greater impact on the diabetic mice (C57/BL6 WT = 256 ± 25%, n = 9; *ob/ob* = 348 ± 20%, n = 10; *db/db* = 384 ± 17%, n = 8; One-way RM ANOVA, $P < 0.05$; *post hoc* Newman Keul's test revealed significant differences between the WT and both types of diabetic mice, $P < 0.05$, but there was no intergroup difference between the *ob/ob* and *db/db* mice, $P > 0.05$; Figure 48; Figure 50). These results indicate that not only is 3α-HSD enzymatic function preserved in mature mice with type-2 diabetic neuropathy, but it may actually be up-regulated. The observation that a similarly large effect of DHP is observed in both *ob/ob* and *db/db* cortical neurones also suggests that the effect is unrelated to the absence of a theoretical direct modulatory effect of leptin on the $GABA_ARs$. Indeed, while the endogenous neurosteroid levels of both the *ob/ob* and *db/db* mice appears to be reduced compared to the WT, the activity of the enzyme 3α-HSD exhibits compensatory up-regulation.

In order to confirm by another method that DHP does not influence cortical $GABA_ARs$ directly, whole-cell voltage-clamp recordings were made in *ob/ob* L2/3 cortical neurones with DHP present in the recording pipette. These recordings were compared against separate control recordings (*i.e.* they were not paired). Analysis of $GABA_AR$-mediated phasic inhibition included the mIPSC amplitude, decay kinetics, charge transfer and frequency.

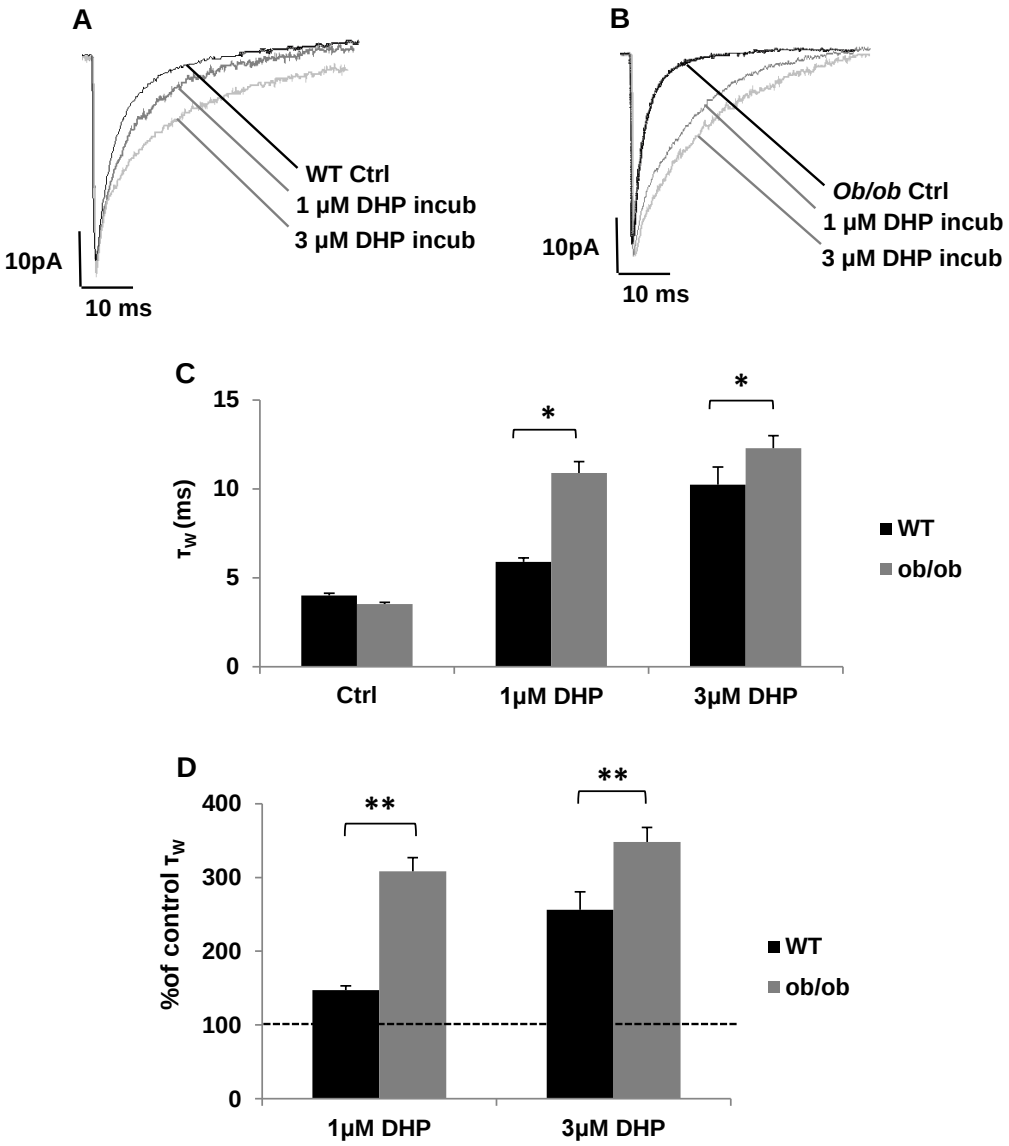

Figure 48 Prolonged exposure (~2 hrs) of mature cortical neurones to DHP (1-3 μM) greatly enhances the function of synaptic $GABA_A$Rs and has an exaggerated effect on the cortical neurones of *ob/ob* mice in comparison to WT mice.

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from a representative control mature WT cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 1 - 3 μM DHP. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from a representative control mature *ob/ob* cortical neurone and from equivalent neurones after ~2 hour brain slice incubation with 1 - 3 μM DHP. **(C)** Histogram illustrating the significant concentration-dependent effect of DHP on the duration of *ob/ob* cortical $GABA_A$Rs mIPSCs (grey bars; *ob/ob* control = 3.5 ± 0.1 ms, n = 25; DHP 1 μM = 10.9 ± 0.6 ms, n = 18; DHP 3 μM = 12.3 ± 0.7 ms, n = 10; One-way ANOVA P <0.05). Note the exaggerated response of the *ob/ob vs.* the WT cortical neurones for 1 μM DHP (C57/BL6 WT 1 μM = 5.9 ± 0.2 ms, n = 14, *ob/ob* 1 μM = 10.9 ± 0.6 ms, n = 18, *P < 0.05) and for 3 μM DHP (C57/BL6 WT 3 μM = 10.2 ± 0.1 ms, n = 9, *ob/ob* 3 μM = 12.3 ± 0.7 ms, n = 10, one-way ANOVA *P < 0.05). **(D)** Histogram illustrating that 1 - 3 μM DHP has an exaggerated effect in *ob/ob* (grey) *vs.* WT (black) mice when the effect is expressed as a percentage of the respective control value (C57/BL6 WT 1 μM = 147 ± 6%, *ob/ob* 1 μM = 308 ± 18%, **P < 0.05; C57/BL6 WT 3 μM = 256 ± 25%, *ob/ob* 3 μM = 348 ± 20%, One-way RM ANOVA **P < 0.05). Ctrl = control; DHP = dihydroxyprogesterone.

The mIPSC decay (τ_W) of L2/3 cortical neurones of *ob/ob* mice was unaffected by the presence of DHP in the recording pipette (*ob/ob* control = 3.5 ± 0.1 ms, n = 25, *ob/ob* 3 μM DHP = 3.6 ± 0.1 ms, n = 6; *ob/ob* 10 μM DHP = 3.7 ± 0.2 ms, n = 4; One-way ANOVA, $P > 0.05$; Figure 49). In view of the fact that the control mIPSC decay times for the WT and diabetic mice are distinct, the data for τ_W were normalised for comparison. Intracellular DHP 3 - 10 μM made no impact on τ_W expressed as a percentage of it's strain representative control (C57/BL6 WT 3 μM = 98 ± 3%, n = 6; *ob/ob* 3 μM = 101 ± 3, n = 6; Two-way RM ANOVA, $P > 0.05$; C57/BL6 WT 10 μM = 108 ± 7%, n = 6; *ob/ob* 10 μM = 104 ± 6%, n = 4; Two-way RM ANOVA, $P > 0.05$; Figure 49). The T_{70} was similarly unaffected in the presence of intracellular DHP. These findings contrast with the significant effects of intracellular allopregnanolone and ganaxolone and are consistent with the idea that DHP is a precursor compound (Figure 49). Considering all the results in this chapter it is difficult to make the case for the alternative hypothesis that $GABA_AR$ sensitivity is increased in *ob/ob* and *db/db* mice.

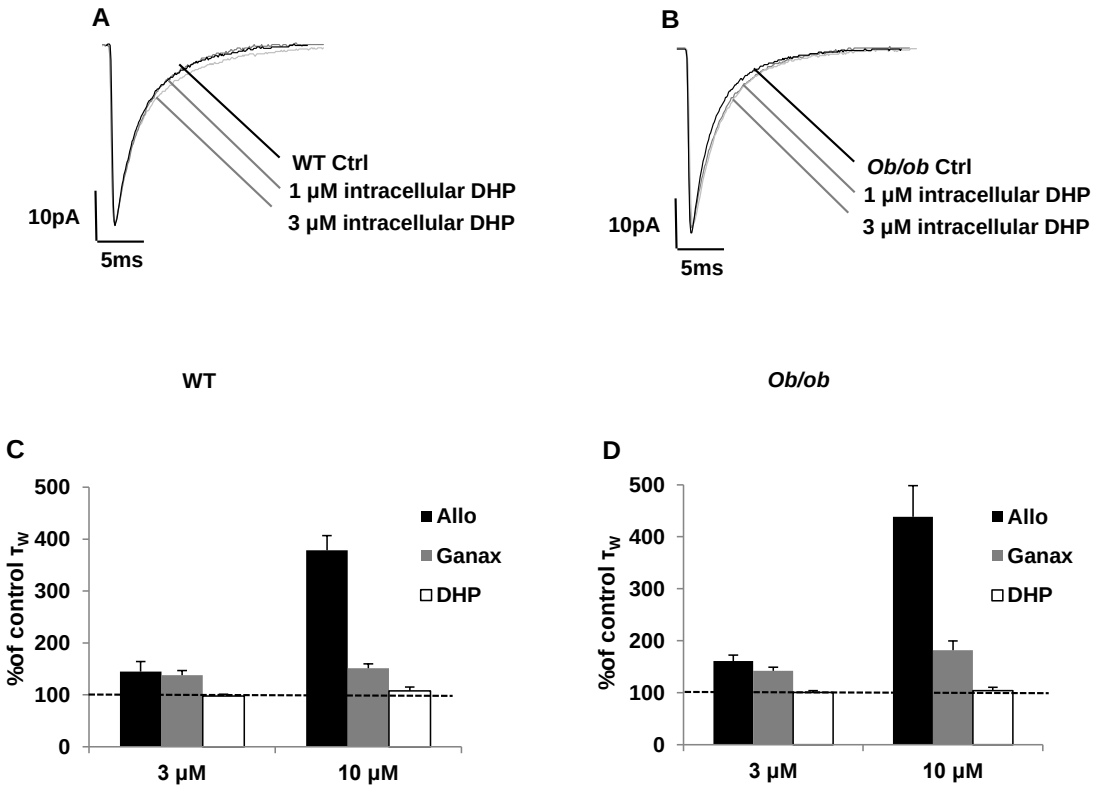

Figure 49 Intracellular DHP has no effect on the function of synaptic $GABA_A$Rs of mature cortical neurones of WT or *ob/ob* mice (in contrast to allopregnanolone & ganaxolone).

(A) Superimposed exemplar $GABA_A$R-mediated mIPSCs from a representative control mature WT cortical neurone and from equivalent neurones with 3 - 10 μM DHP administered intracellularly. **(B)** Superimposed exemplar $GABA_A$Rs mIPSCs from a representative control mature *ob/ob* cortical neurone and from equivalent neurones with 3 - 10 μM DHP administered intracellularly. **(C)** Histogram illustrating the lack of effect of the intracellular application of 3 - 10 μM DHP (white bars) on the duration of *ob/ob* cortical $GABA_A$Rs mIPSC τ_w (One-way ANOVA $P > 0.05$) in contrast to the effectiveness of allopregnanolone and ganaxolone. **(D)** Histogram illustrating the lack of effect of the intracellular application of 3 - 10 μM DHP on the duration of *ob/ob* cortical $GABA_A$Rs mIPSC τ_w expressed as a percentage of the representative control value (One-way RM ANOVA $P > 0.05$) in contrast to the effectiveness of allopregnanolone and ganaxolone. Ctrl = control; Allo = allopregnanolone; Ganax = ganaxolone; DHP = dihydroxyprogesterone.

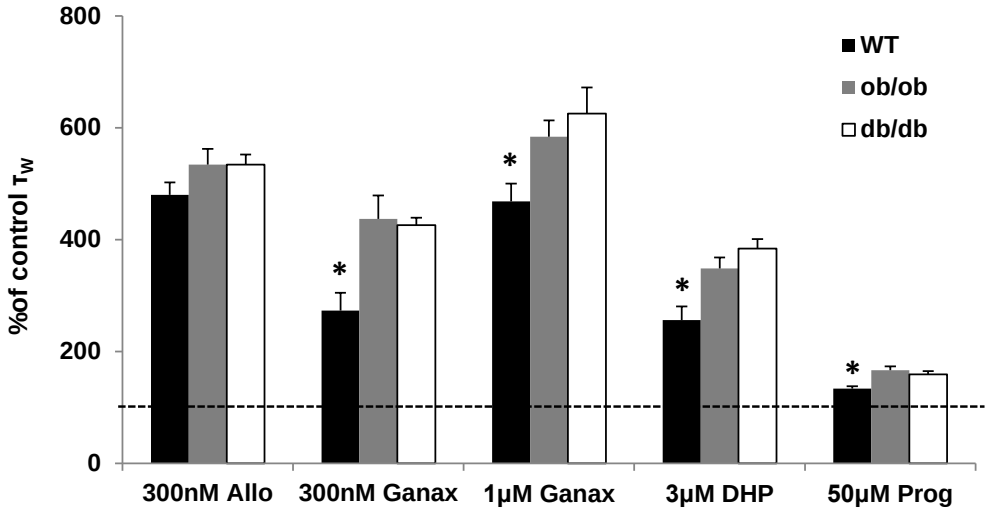

Figure 50 Comparison of the effect of prolonged exposure to several neuroactive steroids on mature cortical neurones of three strains of mice: WT, *ob/ob* and *db/db.*

Histogram summarising the relative differences in the potencies of certain neurosteroidal compounds (allopregnanolone, ganaxolone, DHP and progesterone) in three strains of mice (WT, *ob/ob* and *db/db*) after ~2 hours brain-slice exposure. In view of the fact that the control mIPSC decay times for the WT and "diabetic" mice are distinct, the data for τ_w were normalised for comparison (data included in previous figures and tables). Note that the compounds allopregnanolone and ganaxolone, which modulate the $GABA_AR$ directly, have the greatest potency, while the neurosteroid precursors DHP and progesterone have the least potency. It is also interesting to observe that there is no significant difference in the effect of allopregnanolone on the three strains of mice (C57/BL6 WT = 480 ± 22%, n = 9; *ob/ob* = 534 ± 28%, n = 8; *db/db* = 534 ± 18%, n = 6; One-way RM ANOVA $P > 0.05$). In contrast, the precursor DHP had a greater impact on the diabetic mice (C57/BL6 WT = 256 ± 25%, n = 9; *ob/ob* = 348 ± 20%, n = 10; *db/db* = 384 ± 17%, n = 8; One-way RM ANOVA $^*P < 0.05$. *Post hoc* Newman Keul's test revealed significant differences between the WT and both types of diabetic mice, $^*P < 0.05$, but there was no intergroup difference between the *ob/ob* and *db/db* mice, $P > 0.05$). The precursor progesterone, also had a greater impact on the diabetic mice C57/BL6 WT = 134 ± 4%, n = 9; *ob/ob* = 166 ± 7%, n = 12; *db/db* = 159 ± 6%, n = 6; One-way RM ANOVA $P < 0.05$. *Post hoc* Newman Keul's test revealed significant differences between the WT and both types of diabetic mice, $P < 0.05$, but there was no intergroup difference between the *ob/ob* and *db/db* mice, $P > 0.05$). These data are consistent with the hypothesis that the sensitivity of the cortical $GABA_ARs$ is unchanged in diabetic neuropathy, but that the neurosteroid synthesising enzymes may be upregulated. A surprise finding was that ganaxolone 300 nM (and 1 μM – data not shown) had a greater effect in both strains of diabetic mice compared to WT controls (C57/BL6 WT = 273 ± 32%, n = 10; *ob/ob* = 437 ± 42%, n = 9; *db/db* = 426 ± 13%, n = 4; One-way RM ANOVA $P < 0.05$. *Post hoc* Newman Keul's test revealed significant differences between the diabetic mice and the WT, $^*P < 0.05$, but there was no significant difference between the *ob/ob* and *db/db* mice, $P > 0.05$). This data is consistent with the idea that ganaxolone may modulate the $GABA_AR$ directly, but that it is also a precursor for allopregnanolone, which can be blocked by indometacin incubation treatment, but indometacin has no effect when both drugs are applied acutely *via* the recording pipette.

Chapter 6: Behavioural work

Neuropathic pain places a huge burden on society and the current treatment options available are limited (Bouhassira *et al*., 2008; Dworkin *et al*., 2007; Harstall & Ospina, 2003). Type 2 diabetes mellitus (T2DM) is a major cause of painful peripheral neuropathy and symptoms in humans may include spontaneous sharp, shooting, or burning sensations associated with sensory loss and paradoxical hypersensitivity (Costigan *et al*., 2009; Kehlet *et al*., 2006). Hyperalgesia describes an exaggerated response to a painful stimulus, while allodynia describes an unpleasant or painful response associated with an innocuous stimulus (Merskey & Bogduk, 1994). Both peripheral and central sensitisation are implicated in the development of painful diabetic neuropathy (Fischer & Waxman, 2010). Hypersensitivity of the spinothalamic tract has been described in animal models of type I diabetes (Chen & Pan, 2002, Pertovaara *et al*., 2001). $GABA_AR$s are the major inhibitory receptors in the mammalian nervous system and a reduction of their function is associated with hypersensitivity manifested as allodynia, or hyperalgesia (Munro *et al*., 2009; von Hehn *et al*., 2012; Yaksh, 1989; Zeilhofer, 2008). Conversely, endogenous neurosteroid production may increase in response to peripheral inflammatory pain, thus exerting a putative compensatory analgesic effect (Poisbeau *et al*., 2005). In addition, $GABA_AR$ ligands that target the $\alpha 2$ and $\alpha 3$ subunits of the receptor specifically are effective against inflammatory and neuropathic pain without side effects such as sedation, tolerance and ataxia (Knabl *et al*., 2008).

The *ob/ob* mouse develops a predictable neuropathic phenotype by 8 weeks of age comparable to that observed in humans with T2DM, thus making it a useful model (Drel *et al.,* 2006; Latham *et al.,* 2009; Lindstrom, 2009; Vareniuk *et al.,* 2007). Drel *et al.,* reported that *ob/ob* mice develop mechanical hypersensitivity and thermal hyposensitivity, while Latham *et al.,* reported both mechanical and thermal hypersensitivity (Drel *et al.,* 2006; Latham *et al.,* 2009). The authors of these papers used paw withdrawal latency to a radiant heat source and paw withdrawal threshold to von Frey (vF) filaments (Drel *et al.,* 2006; Latham *et al.,* 2009).

In the previous results chapters (3-5), I have described the impact of the endogenous neurosteroid tone on synaptic $GABA_AR$ function in pain pathway neurones of WT and diabetic mice. In particular, I discovered that the endogenous neurosteroid tone is reduced in layer 2/3 cortical neurones of mature *ob/ob* mice (aged P60-75 or ~9-11 weeks). In addition, mIPSCs mediated by layer 2/3 cortical $GABA_ARs$ of *ob/ob* mice also exhibited an increased response to ganaxolone and DHP when compared to mIPSCs recorded from WT cortical neurones. These findings suggest that a decreased neurosteroid tone may be implicated in neuropathic hypersensitivity exhibited by these mice. Therefore, it is reasonable to consider that the administration of exogenous neurosteroids could potentially be analgesic in *ob/ob* mice. Indeed, allopregnanolone, DHP and progesterone have had promising effects in the prevention of chemotherapy-induced neuropathic pain (Meyer *et al*., 2010; 2011).

In my experiments, the tail flick test and vF filaments were used to determine thermal and mechanical nociceptive thresholds respectively in WT and *ob/ob* mice aged ~9-11 weeks (P60-75; D'Armour & Smith, 1941, Mogil *et al.,* 2009). In addition, acetone was applied topically in order to induce a rapid cooling effect, which may be uncomfortable in hypersensitive states (Flatters & Bennett, 2004, Gauchan *et al.,* 2009, Meyer *et al.,* 2011). I also used the rotarod test to assess sensorimotor coordination in both strains of mice.

6.1 *ob/ob* mice have impaired sensorimotor coordination in comparison to WT mice.

ob/ob mice have reduced motor and sensory nerve conduction velocities in comparison to WT mice (associated with reduced nerve fibre density), which impairs balance and coordination (Drel *et al.,* 2006; Vareniuk *et al.,* 2007). The rotarod test comprises an elevated rotating cylinder upon which a rodent is placed. In order to avoid falling off the rotarod, the mouse must keep moving; hence it is a test of forced motor activity (Jones & Roberts, 1968; Pritchett & Mulder, 2003). To remain on the rotarod while it accelerates at a set rate the mouse requires balance

and coordination. The rotarod may therefore be used to test motor function after the administration of novel agents as part of the drug development process or to characterise neurodegenerative conditions (Pallier *et al.,* 2009; Shiotsuki *et al.,* 2010). WT or *ob/ob* mice were placed on the rotarod and the amount of time that they remained on the accelerating rod was recorded, the maximum of which was 300 seconds. The *ob/ob* mice were able to remain on the rotarod for a significantly shorter time than were WT mice (WT = 216 ± 14 s, n = 15; *ob/ob* = 21 ± 5 s, n = 15; Mann-Whitney Rank Sum test, $P < 0.001$; Figure 51 A). The impaired rotarod performance of the *ob/ob* mouse is consistent with a previous report (Mayers *et al.,* 2009).

6.2 *ob/ob* mice exhibit prolonged tail withdrawal from noxious heat in comparison to WT mice.

The tail flick test was first described using a light bulb to apply a heat stimulus to the mouse's tail and is now one of the standard tests of thermal nociceptive pain (D'Amour & Smith, 1941; Morgan *et al.,* 2006). Here, the mouse's tail was placed into a bath filled with hot water and the time taken for tail withdrawal was recorded. A maximum withdrawal latency of 15 seconds was enforced to minimise the possibility of tissue damage. This approach was used to characterise the response of the WT and the *ob/ob* mouse to three distinct temperatures: 46 ^{o}C, 48 ^{o}C and 50 ^{o}C. At 48 ^{o}C and 50 ^{o}C *ob/ob* mice exhibited significantly longer tail withdrawal latencies compared to wild type mice (WT 48 ^{o}C = 5.4 ± 0.8s; *ob/ob* 48 ^{o}C = 7.5 ± 0.9s, $P < 0.05$; WT 50 ^{o}C = 2.5 ± 0.2*s*; *ob/ob* 50 ^{o}C = 5.0 ± 1s, $P < 0.05$; Figure 51). By contrast, there was no significant difference with a water temperature of 46 ^{o}C (WT = 12.1 ± 1s; *ob/ob* = 14.1 ± 0.6s, n = 20 per group, Mann-Whitney Rank Sum test, $P > 0.05$; Figure 51). These findings are consistent with the phenomenon of thermal hypoalgesia in *ob/ob* mice reported by Drel *et al.,* (2006), but conflict with the thermal hyperalgesia reported by Latham *et al.,* (2009).

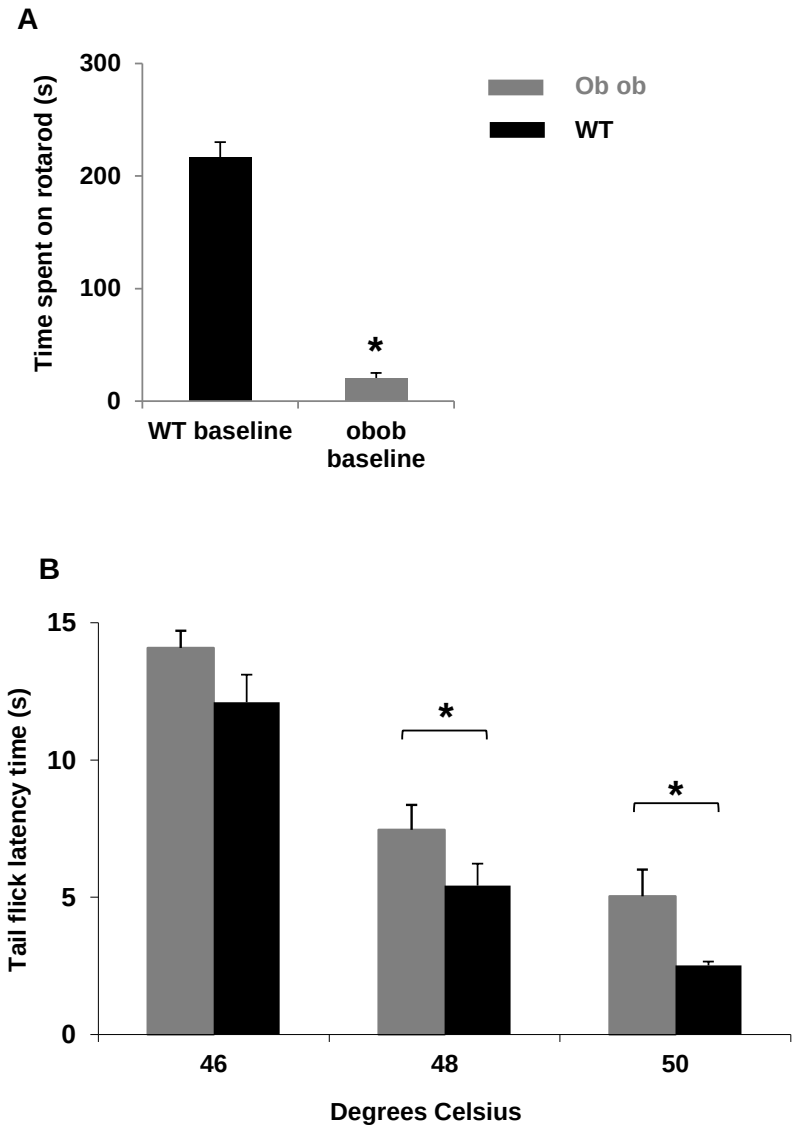

Figure 51 *Ob/ob* mice have impaired sensorimotor coordination and exhibit prolonged tail withdrawal from noxious heat in comparison to WT mice.

(A) Histogram illustrating the dramatic impairment of sensorimotor function exhibited by mature *ob/ob* mice. The *ob/ob* mice were able to remain on the accelerating rotarod for a significantly shorter time than were WT mice (WT = 216 ± 14 s, n = 15; *ob/ob* = 21 ± 5 s, n = 15; Mann-Whitney Rank Sum test $P < 0.001$). **(B)** Histogram illustrating that mature *ob/ob* mice have a delayed response to thermal noxious stimuli in comparison to age-matched WT mice. (WT 48 ^{o}C = 5.4 ± 0.8s; *ob/ob* 48 ^{o}C = 7.5 ± 0.9s, $P < 0.05$; WT 50 ^{o}C = 2.5 ± 0.2s; *ob/ob* 50 ^{o}C = 5.0 ± 1s, $P < 0.05$). There was no significant difference at the less noxious temperature of 46 ^{o}C (WT = 12.1 ± 1s; *ob/ob* = 14.1 ± 0.6s, n = 20 per group, Mann-Whitney Rank Sum test $P > 0.05$). A maximum withdrawal latency of 15 seconds was enforced to minimise the possibility of tissue damage.

The discrepancy is considered further in the Discussion: Section 7.11 and means that the tail flick test is not suitable for investigating potential analgesic, or antihyperalgesic effects of drugs such as neurosteroids in *ob/ob* mice. However, these data indicate that 50 ^{o}C is an appropriate temperature to test for noxious heat and to determine the analgesic potential of specific drugs in WT mice.

6.3 Ganaxolone increases tail withdrawal latency in WT mice.

The spinal (intrathecal) administration of ganaxolone has recently been shown to reduce thermal nociception using the tail flick test, but systemic (intraperitoneal) administration has not been investigated (Asiedu *et al.,* 2012). Other structurally related GABA-active steroids also exhibit analgesic effects (Poisbeau *et al.,* 2005, Charlet *et al.,* 2008, Meyer *et al.,* 2008; 2010; 2011). The tail flick test is a useful tool for assessing thermal nociception, therefore the analgesic effect of ganaxolone was assessed at three different doses. Ganaxolone required to be solubilised with hydroxypropyl β-cyclodextrin (β-CD) prior to intraperitoneal injection (Besheer *et al.,* 2010; Carter *et al.,* 1997; Reddy *et al.,* 2010). The cyclodextrins are effective solubilising agents and do not cause a localised inflammatory response at the injection site, which is a relatively common side effect of some alternative solvents such as DMSO (Loftsson & Brewster, 1996; Willson *et al.,* 1965).

There were no significant differences in the baseline withdrawal latencies in response to a noxious thermal stimulus (50 ^{o}C) between the four groups of mice used to examine the effects of ganaxolone (β-CD = 1.9 ± 0.3 s, ganax 3 mg/kg = 1.1 ± 0.1 s, 10 mg/kg = 1.5 ± 0.1 s, 30 mg/kg = 2.8 ± 0.7 s, $P > 0.05$) and the solubilising vehicle β-CD also had no effect (β-CD 30 mins = 2.0 ± 0.1 s, β-CD 60 mins = 2.5 ± 0.2 s, β-CD 120 mins = 2.7 ± 0.3 s, $P > 0.05$). Ganaxolone induced a dose-dependent prolongation of tail withdrawal latency in WT mice after 30 minutes (ganax 3 mg/kg 30 mins = 1.5 ± 0.2 s, 10 mg/kg 30 mins = 6.1 ± 1.8 s, 30 mg/kg 30 mins = 6.0 ± 2 s, $P < 0.05$) and this effect lasted more than 90 minutes (ganax 3 mg/kg 60 mins = 2.1 ± 0.3 s, 10 mg/kg 60 mins = 5.2 ± 1.7 s, 30 mg/kg 60 mins = 8.8 ± 2 s, $P < 0.05$; ganax 3 mg/kg 120 mins = 3.8 ± 1.3 s, 10 mg/kg 120 mins = 7.3 ± 2.1 s, 30 mg/kg 120 mins = 11.5 ± 1.8 s, n = 10 per group, Kruskall Wallis One-way ANOVA,

on ranks $P < 0.05$; Figure 52). These data suggest that ganaxolone exhibits a dose-dependent analgesic effect in a test of thermal nociception in WT mice. This finding is consistent with the intrathecal administration of ganaxolone (Asiedu *et al.,* 2012) and also other reports of the analgesic effects of similar neurosteroids in rats in the setting of post-chemotherapy neuropathy (Meyer *et al.,* 2010; 2011). However, during the experiments it was noted that the mice appeared sedated with ganaxolone 30 mg/kg (but not the lower doses). Therefore rotarod experiments were carried out with ganaxolone to determine if the apparent analgesic effect may in fact be due to sedation, or sensorimotor impairment.

6.4 High doses of ganaxolone impair rotarod performance of WT mice.

The accelerating rotarod test was used to assess forced motor activity before and after the intraperitoneal administration of ganaxolone at three doses. Only the highest dose of ganaxolone significantly impaired the performance of WT mice on the rotarod. There was no significant difference in the baseline rotarod performance of the four groups in the study (β-CD baseline = 215 ± 28 s, ganax 5 mg/kg baseline = 155 ± 16 s, 10 mg/kg baseline = 216 ± 24 s, 30 mg/kg baseline = 211 ± 40 s, $P > 0.05$) and the solubilising vehicle β-CD had no effect on rotarod performance (β-CD 15 mins = 208 ± 28s, β-CD 30mins = 243 ± 27s, β-CD 60mins = 182 ± 35s, β-CD 120mins = 208 ± 45s, $P > 0.05$; Figure 53). However, at 15, 30 and 60 minutes post-injection the highest dose of ganaxolone (30 mg/kg) significantly impaired performance of WT mice on the rotarod (ganax 5 mg/kg 15 mins = 165 ± 33 s, 10 mg/kg 15 mins = 136 ± 20 s, 30 mg/kg 15 mins = 42 ± 33 s, $P < 0.05$; ganax 5 mg/kg 30 mins = 211 ± 28 s, 10 mg/kg 30 mins = 161 ± 30 s, 30 mg/kg 30 mins = 54 ± 41 s, $P < 0.05$; ganax 5 mg/kg 60 mins = 172 ± 32 s, 10 mg/kg 60 mins = 196 ± 21 s, 30 mg/kg 60 mins = 36 ± 26 s, $P < 0.05$). The effect of ganaxolone (30 mg/kg) was no longer apparent at 120 minutes post-injection (ganax 5 mg/kg 120 mins = 220 ± 17 s, 10 mg/kg 120 mins = 221 ± 12 s, 30 mg/kg 120 mins = 155 ± 65 s, n = 5 per group, Kruskall Wallis One-way ANOVA, on ranks $P > 0.05$; Figure 53).

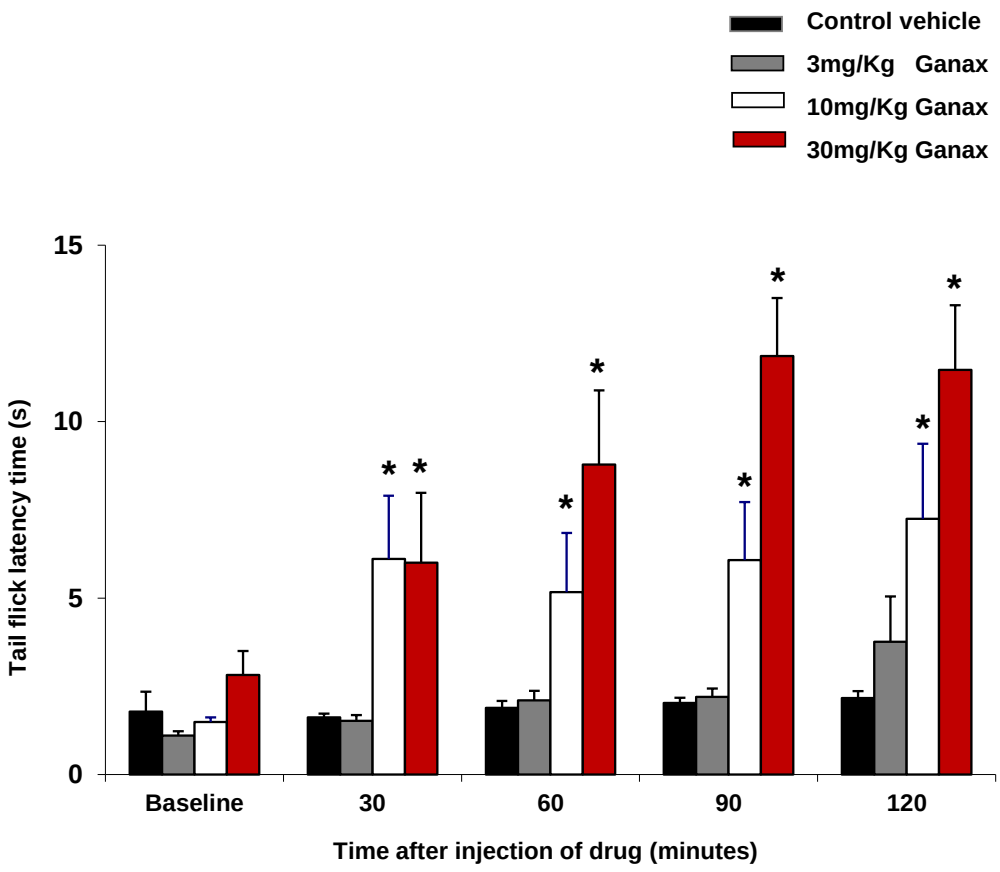

Figure 52 Ganaxolone increases the latency for tail withdrawal from noxious heat in WT mice.

Histogram illustrating that ganaxolone induced a dose-dependent prolongation of tail withdrawal latency in WT mice after 30 minutes (ganax 3 mg/kg 30 mins = 1.5 ± 0.2 s, 10 mg/kg 30 mins = 6.1 ± 1.8 s, 30 mg/kg 30 mins = 6.0 ± 2 s, $P < 0.05$) and this effect lasted more than 90 minutes (ganax 3 mg/kg 60 mins = 2.1 ± 0.3 s, 10 mg/kg 60 mins = 5.2 ± 1.7 s, 30 mg/kg 60 mins = 8.8 ± 2 s, $P < 0.05$; ganax 3 mg/kg 120 mins = 3.8 ± 1.3 s, 10 mg/kg 120 mins = 7.3 ± 2.1 s, 30 mg/kg 120 mins = 11.5 ± 1.8 s, n = 10 per group, $P < 0.05$). The solubilising vehicle β-CD had no effect (β-CD 30 mins = 2.0 ± 0.1 s, β-CD 60 mins = 2.5 ± 0.2 s, β-CD 120 mins = 2.7 ± 0.3 s; Kruskall Wallis One-Way ANOVA on ranks $P > 0.05$).

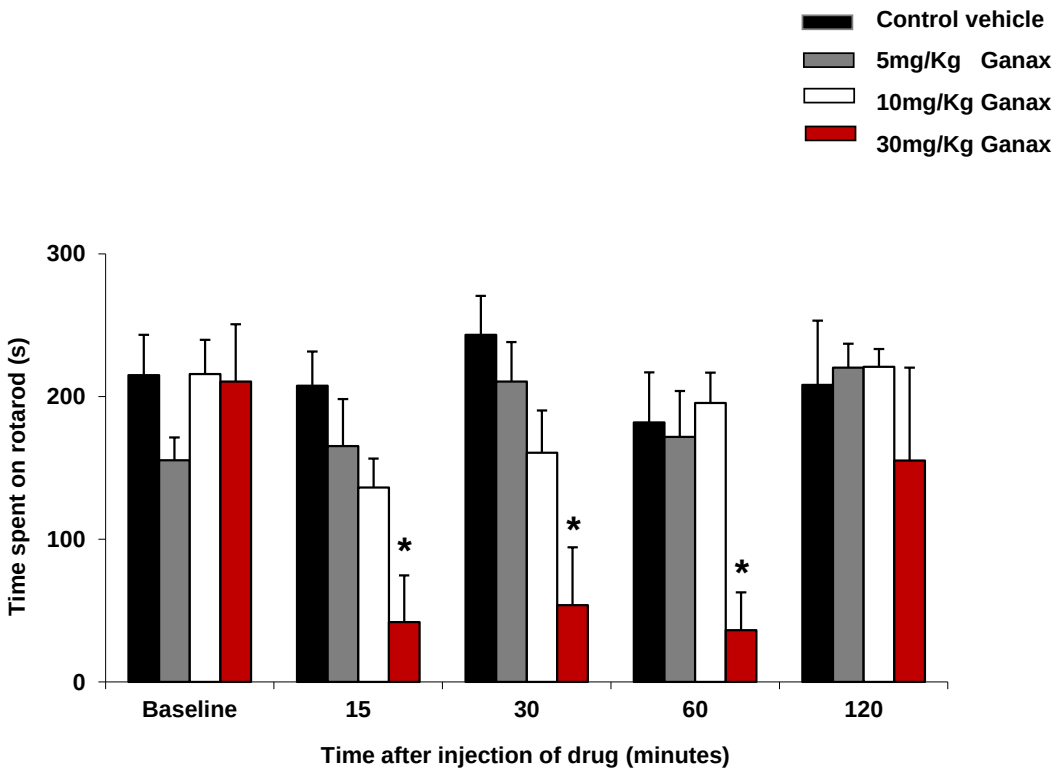

Figure 53 The highest dose of ganaxolone impairs the rotarod performance of WT mice.

Histogram showing that at 15, 30 and 60 minutes post-injection the highest dose of ganaxolone (30 mg/kg) significantly impaired performance of WT mice on the rotarod (ganax 5 mg/kg 15 mins = 165 ± 33 s, 10 mg/kg 15 mins = 136 ± 20 s, 30 mg/kg 15 mins = 42 ± 33 s, $P < 0.05$; ganax 5 mg/kg 30 mins = 211 ± 28 s, 10 mg/kg 30 mins = 161 ± 30 s, 30 mg/kg 30 mins = 54 ± 41 s, $P < 0.05$; ganax 5 mg/kg 60 mins = 172 ± 32 s, 10 mg/kg 60 mins = 196 ± 21 s, 30 mg/kg 60 mins = 36 ± 26 s, $P < 0.05$). The effect of ganaxolone (30 mg/kg) was no longer apparent at 120 minutes post-injection (ganax 5 mg/kg 120 mins = 220 ± 17 s, 10 mg/kg 120 mins = 221 ± 12 s, 30 mg/kg 120 mins = 155 ± 65 s, n = 5 per group, $P > 0.05$). The solubilising vehicle β-CD had no effect on rotarod performance (β-CD 15 mins = 208 ± 28s, β-CD 30mins = 243 ± 27s, β-CD 60mins = 182 ± 35s, β-CD 120mins = 208 ± 45s, Kruskall Wallis One-Way ANOVA on ranks $P > 0.05$).

Taken in conjunction with the tail flick data, these data suggest that ganaxolone exhibits an analgesic effect at a dose of 10 mg/kg but only impairs rotarod performance in WT mice at higher doses such as 30 mg/kg. In addition, the dose-dependent effect on rotarod performance is consistent with the literature (Carter *et al.,* 1997).

6.5 *ob/ob* mice exhibit cold allodynia in comparison to WT mice.

Increased sensitivity to cold stimuli is a common symptom associated with neural injury. The cooling sensation associated with the evaporation of topically applied acetone may induce a temporary noxious stimulus. Acetone has therefore been used to elicit cold allodynia in animals with chemotherapy-induced neuropathic hypersensitivity (Flatters & Bennett, 2004, Gauchan *et al.,* 2009, Meyer *et al.,* 2011).

In my study, mice were placed onto an elevated grid-platform, a drop of acetone was applied to the ventral (plantar) surface of the animal's hindpaw and the mouse was observed for 20 seconds. The following scoring system was used: (0 = No response; 1 = Quick withdrawal, flick or stamp of the paw only; 2 = Prolonged/repeated flicking of the paw and/or abnormal hind limb posture/limping; 3 = The same as above but also licking of the ventral surface of the affected paw) (Flatters & Bennett, 2004). The *ob/ob* mouse exhibited a significantly greater response to the application of topical acetone (WT acetone = 0.96 ± 0.1, n = 20; *ob/ob* acetone = 2.2 ± 0.1, n = 20; Mann-Whitney Rank Sum test, $P < 0.05$; Figure 54). This result is consistent with the presence of cold allodynia in *ob/ob* mice. However, this test proved to be problematic for further study because in subsequent pilot experiments repeated exposure of *ob/ob* mice to acetone (required to evaluate the subsequent effects of neurosteroids) led to a loss of the allodynia response (pre-vehicle = 2.2 ± 0.2, post-vehicle = 1.7 ± 0.2, n = 5; Mann-Whitney Rank Sum test $P < 0.05$).

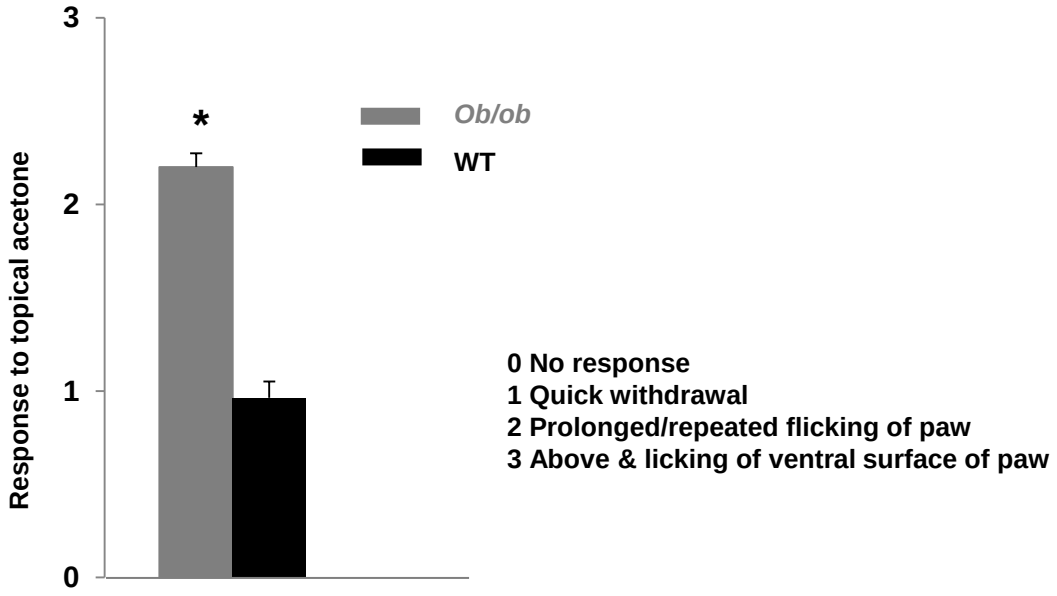

Figure 54 *Ob/ob* mice exhibit cold allodynia in comparison to WT mice.

Histogram illustrating that *ob/ob* mice exhibit cold allodynia in comparison to WT mice. The mice were placed onto an elevated grid-platform, a drop of acetone was applied to the ventral (plantar) surface of the animal's hindpaw and the mouse was observed for 20 seconds. The following scoring system was used: (0 = No response; 1 = Quick withdrawal, flick or stamp of the paw only; 2 = Prolonged/repeated flicking of the paw and/or abnormal hind limb posture/limping; 3 = The same as for score 2 but with the addition of licking of the ventral surface of the affected paw). The *ob/ob* mouse exhibited a significantly greater response to the application of topical acetone (WT acetone = 0.96 ± 0.1, n = 20 mice; *ob/ob* acetone = 2.2 ± 0.1, n = 20 mice, Mann-Whitney Rank Sum test $P < 0.05$).

6.6 *ob/ob* mice exhibit mechanical hypersensitivity in comparison to WT mice.

The calibrated series of von Frey (vF) filaments are standard equipment for assessing mechanical nociceptive thresholds in animal models and also in humans (Mogil *et al.,* 2009; Pearce, 2006). vF filaments have previously been employed *via* the up-down method to demonstrate that the *ob/ob* mouse exhibits mechanical hypersensitivity by the age of P60 (Drel *et al.,* 2006; Latham *et al.,* 2009). I carried out pilot studies to determine the optimal four filaments to be used in WT mice; these were the 0.16 g, 0.4 g, 0.6 g and 1 g filaments. Mice were placed onto an elevated grid-platform and each vF filament was applied to the plantar surfaces of the left and right hind paws five times. The number of withdrawals out of a maximum of ten was recorded for a particular filament in each mouse.

The 0.16 g and 0.4 g vF filaments elicit withdrawal responses on 25% and 40% of occasions respectively in WT mice and were therefore considered to be mild stimuli. If the *ob/ob* mouse has an exaggerated response to these filaments this would be consistent with allodynia. The 0.6 g vF filament elicited a response on over 60% of occasions in the WT mouse, therefore it is considered painful and if the *ob/ob* mouse had an exaggerated response to this filament it would be consistent with hyperalgesia (Merskey & Bogduk, 1994). The 1 g vF filament elicited a withdrawal response on approximately 90% of occasions, therefore it is considered to be a clear test of mechanical nociceptive pain in the WT mouse. This method was adapted from work published in rats with neuropathic sensitisation (Meyer *et al.,* 2011).

The *ob/ob* mice responded significantly more frequently than the WT mice to the 0.16 g, 0.4 g and 0.6 g filaments (WT 0.16 g = 30.9 ± 3.2%, n = 33; *ob/ob* 0.16 g = 41.8 ± 3.9%, $P < 0.05$; WT 0.4 g = 56.4 ± 3.7%; *ob/ob* 0.4 g = 68.8 ± 3.8%, $P < 0.05$; WT 0.6 g = 69.1 ± 3.5%; *ob/ob* 0.6 g = 85.2 ± 2.5%, $P < 0.05$; Figure 55). By contrast, there was no significant difference in the frequency of responses to the 1 g vF filament (WT = 84.8 ± 2.9%, n = 33; *ob/ob* = 92.7 ± 1.7%, n = 33 per group; Mann-Whitney Rank Sum test, $P > 0.05$). These results confirm the presence of

mechanical hypersensitivity in the *ob/ob* mouse, which is consistent with reports in the literature (Drel *et al.,* 2006; Latham *et al.,* 2009).

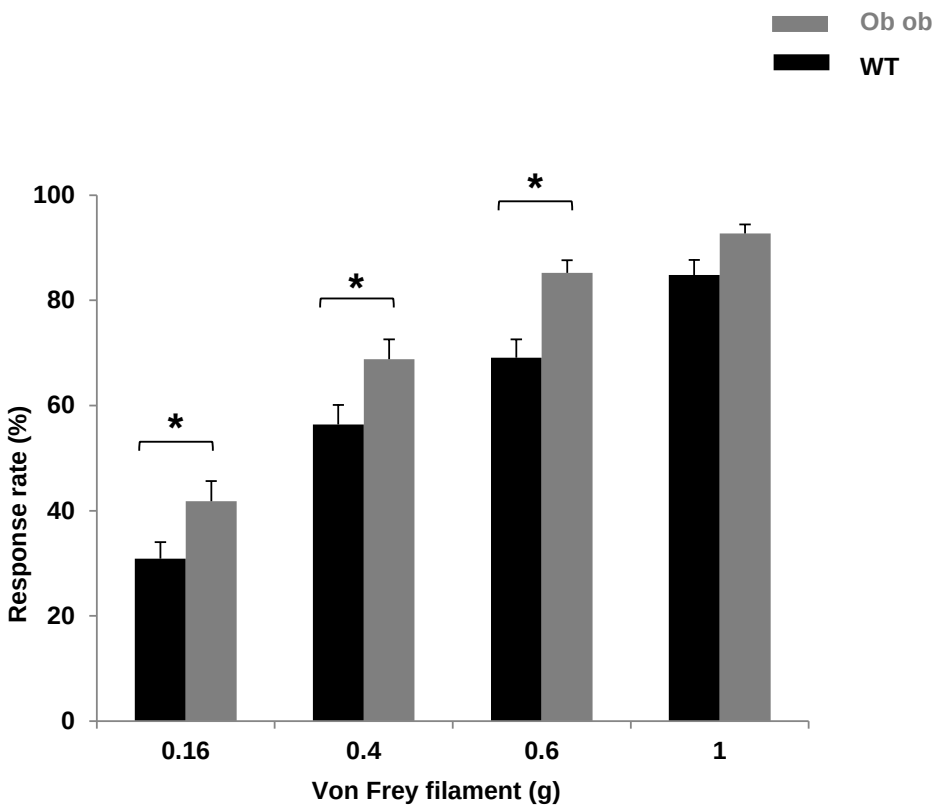

Figure 55 *Ob/ob* mice exhibit mechanical hypersensitivity in comparison to WT mice.

Histogram illustrating that *ob/ob* mice exhibit mechanical hypersensitivity in comparison to WT mice. The mice were placed onto an elevated grid-platform and each vF filament (0.16 g – 1 g) was applied to the plantar surfaces of the left and right hind paws five times. The number of withdrawals out of a maximum of ten was recorded for a particular filament in each mouse. The *ob/ob* mice responded significantly more frequently than the WT mice to the 0.16 g, 0.4 g and 0.6 g filaments (WT 0.16 g = 30.9 ± 3.2%, n = 33; *ob/ob* 0.16 g = 41.8 ± 3.9%, $P < 0.05$; WT 0.4 g = 56.4 ± 3.7%; *ob/ob* 0.4 g = 68.8 ± 3.8%, $P < 0.05$; WT 0.6 g = 69.1 ± 3.5%; *ob/ob* 0.6 g = 85.2 ± 2.5%, $P < 0.05$). By contrast, there was no significant difference in the frequency of responses to the 1 g vF filament (WT = 84.8 ± 2.9%, n = 33; *ob/ob* = 92.7 ± 1.7%, n = 33 per group; Mann-Whitney Rank Sum test $P > 0.05$). These results confirm the presence of mechanical hypersensitivity in the *ob/ob* mouse.

6.7 Ganaxolone reduces mechanical hypersensitivity in *ob/ob* mice and reduces mechanical nociceptive pain in both WT and *ob/ob* mice.

β-CD vehicle alone or β-CD plus ganaxolone was administered *via* the intraperitoneal route. The same series of vF filaments were used as described for the comparison of mechanical sensitivity of WT and *ob/ob* mice (0.16 g, 0.4 g, 0.6 g and 1 g). After control recordings were obtained, the drug was injected and the assay was repeated one hour later.

The β-CD vehicle had no impact on the response to any of the vF filaments in WT mice (0.16 g pre β-CD = 36 ± 6%, 0.16 g post β-CD = 46 ± 5%, $P > 0.05$; 0.4 g pre = 70 ± 6%, 0.4 g post = 68 ± 7%, P > 0.05; 0.6 g pre = 77 ± 6%, 0.6 g post = 74 ± 6%, $P > 0.05$; 1 g pre = 90 ± 5%, 1 g post = 88 ± 6%, $P > 0.05$) or *ob/ob* mice (0.16 g pre β-CD = 43 ± 6%, 0.16 g post β-CD = 39 ± 5%, $P > 0.05$; 0.4 g pre = 63 ± 7%, 0.4 g post = 53 ± 5%, $P > 0.05$; 0.6 g pre = 83 ± 5%, 0.6 g post = 74 ± 5%, $P > 0.05$; 1 g pre = 89 ± 5%, 1 g post = 84 ± 4%, $P > 0.05$; Figure 56).

Ganaxolone (10 mg/kg) had no impact on the response to the 0.16 g, 0.4 g or 0.6 g vF filaments in the WT mouse (0.16 g pre ganax = 31 ± 6%, 0.16 g post ganax = 42 ± 6%, 0.4 g pre = 61 ± 6%, 0.4 g post = 57 ± 7%, 0.6 g pre = 79 ± 4%, 0.6 g post = 65 ± 6%, $P > 0.05$) but ganaxolone did reduce the response of WT mice to the 1.0 g vF filament (pre ganax = 94 ± 3%, post ganax = 82 ± 5%, n = 10 per group, Wilcoxon signed rank test (before & after), $P < 0.05$; Figure 57). These data suggest that ganaxolone is analgesic for mechanical nociceptive pain in WT mice. By definition, WT mice do not exhibit allodynia or hyperalgesia under normal conditions; therefore it is perhaps unsurprising that these drugs did not impact on the response to the smaller vF filaments.

Ganaxolone had no impact on the response rate to the 0.16 g vF filament in *ob/ob* mice (pre ganax = 42 ± 7%, post ganax = 38 ± 8%, $P > 0.05$). In contrast, ganaxolone reduced the response rate of *ob/ob* mice to the 0.4 g, 0.6 g and 1 g vF filaments (0.4 g pre ganax = 66 ± 7%, 0.4 g post ganax = 49 ± 6%, $P < 0.05$; 0.6 g pre = 82 ± 5%, 0.6 g post = 59 ± 7%, $P < 0.05$; 1 g pre = 92 ± 4%, 1 g post = 74 ± 8%, n = 10 per group, Wilcoxon signed rank test (before & after), $P < 0.05$; Figure 57).

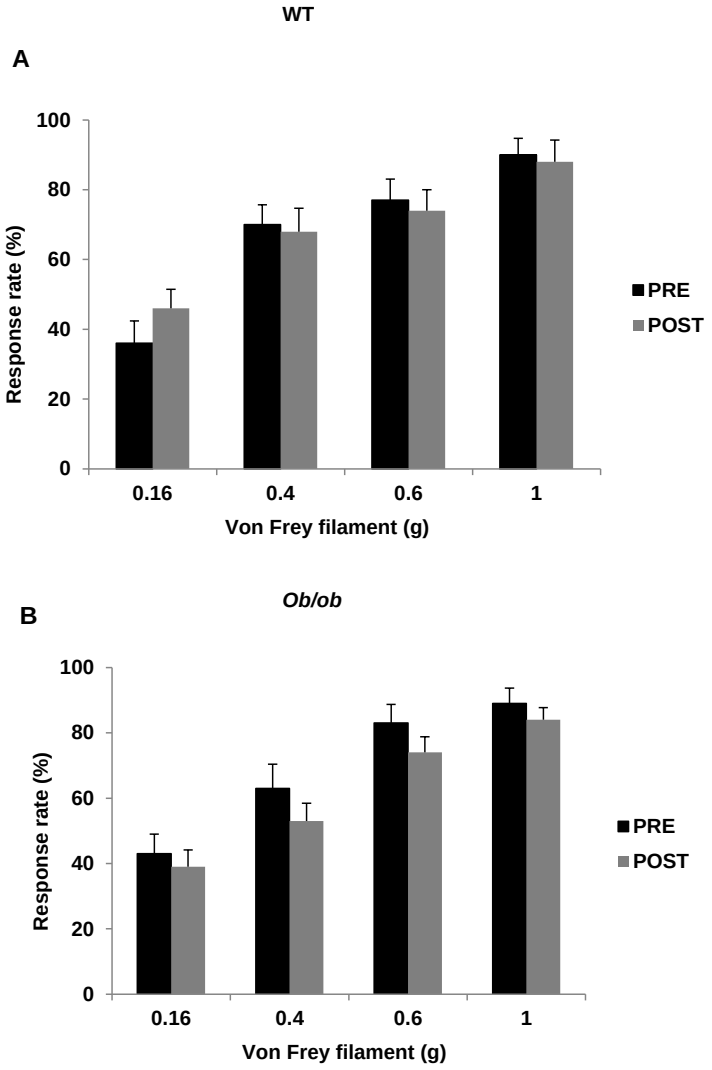

Figure 56 Vehicle has no significant effect in *ob/ob* mice or WT mice.

(A & **B)** Histograms illustrating (for control purposes) that the β-CD vehicle had no impact on the response to any of the vF filaments in WT mice (0.16 g pre β-CD = 36 ± 6%, 0.16 g post β-CD = 46 ± 5%, $P > 0.05$; 0.4 g pre = 70 ± 6%, 0.4 g post = 68 ± 7%, $P > 0.05$; 0.6 g pre = 77 ± 6%, 0.6 g post = 74 ± 6%, $P > 0.05$; 1 g pre = 90 ± 5%, 1 g post = 88 ± 6%, $P > 0.05$) or *ob/ob* mice (0.16 g pre β-CD = 43 ± 6%, 0.16 g post β-CD = 39 ± 5%, $P > 0.05$; 0.4 g pre = 63 ± 7%, 0.4 g post = 53 ± 5%, $P > 0.05$; 0.6 g pre = 83 ± 5%, 0.6 g post = 74 ± 5%, $P > 0.05$; 1 g pre = 89 ± 5%, 1 g post = 84 ± 4%, $P > 0.05$).

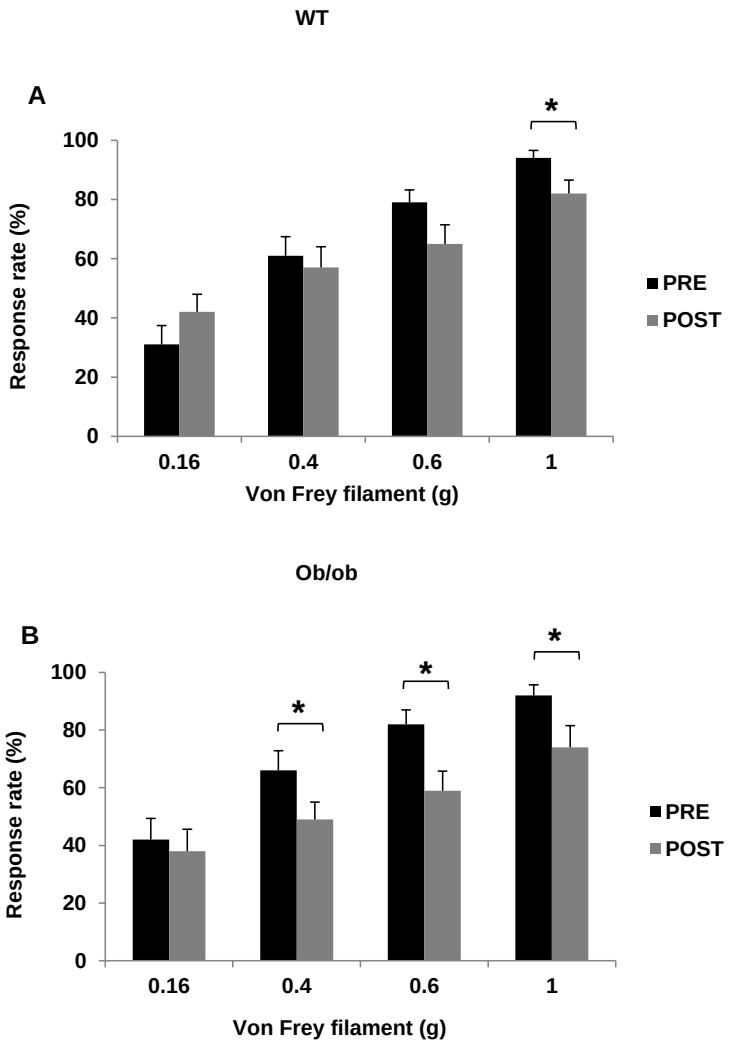

Figure 57 Ganaxolone reduces mechanical hypersensitivity in *ob/ob* mice and reduces mechanical nociceptive pain in both WT and *ob/ob* mice.

(A) Histogram illustrating that ganaxolone had no impact on the response to the 0.16 g, 0.4 g or 0.6 g vF filaments in WT mice (0.16 g pre ganax = 31 ± 6%, 0.16 g post ganax = 42 ± 6%, 0.4 g pre = 61 ± 6%, 0.4 g post = 57 ± 7%, 0.6 g pre = 79 ± 4%, 0.6 g post = 65 ± 6%, $P > 0.05$) but ganaxolone did reduce the response of WT mice to the 1.0 g vF filament (pre ganax = 94 ± 3%, post ganax = 82 ± 5%, n = 10 per group, Wilcoxon signed rank test (before & after) $P < 0.05$). **(B)** Histogram illustrating that ganaxolone had no impact on the response to the 0.16 g vF filament the *ob/ob* mouse (pre-ganax = 42 ± 7%, post-ganax = 38 ± 8%, $P > 0.05$). In contrast, ganaxolone reduced the response of the *ob/ob* mouse to the 0.4 g, 0.6 g and 1 g vF filaments (0.4 g pre-ganax = 66 ± 7%, 0.4 g post-ganax = 49 ± 6%, $P < 0.05$; 0.6 g pre = 82 ± 5%, 0.6 g post = 59 ± 7%, $P < 0.05$; 1 g pre = 92 ± 4%, 1 g post = 74 ± 8%, n = 10 per group, Wilcoxon signed rank test (before & after) $P < 0.05$).

As described earlier, *ob/ob* mice have exaggerated baseline response rates to the 0.16 g, 0.4 g and 0.6 g vF filaments in comparison to WT mice (Figure 55). Therefore, *ob/ob* mice could be considered to have mechanical allodynia and mechanical hyperalgesia. Ganaxolone reduced the response rates of *ob/ob* mice to the 0.4 g vF filament, which is consistent with the idea that these neurosteroids may reduce mechanical allodynia. Ganaxolone also reduced the response rates of *ob/ob* mice to the 0.6 g vF filament, which is consistent with the idea that it reduces mechanical hyperalgesia. As described earlier, the 1.0 g vF filament induces a withdrawal response on ~85% of occasions in WT mice and ~93% in *ob/ob* mice (Figure 55) and may therefore be considered as an unambiguous test of mechanical nociceptive pain. Taken as a whole, these results show that ganaxolone was effective for mechanical nociceptive pain in both strains of mice.

As described in Chapters 4 and 5, DHP (the precursor for allopregnanolone) induced a dramatic prolongation of $GABA_AR$ mIPSC decay time in layer 2/3 cortical neurones of WT mice. Interestingly, DHP (in common with ganaxolone) had an exaggerated effect on cortical $GABA_AR$ mIPSCs of *ob/ob* mice compared to WT mice. Loss of $GABA_AR$-mediated inhibition is associated with hypersensitivity (Munro *et al*., 2009; von Hehn *et al*., 2012; Yaksh, 1989; Zeilhofer, 2008), therefore DHP (which prolongs $GABA_AR$ mIPSCs) was also used to investigate mechanical nociception. DHP had no impact on the rates of response to any of the vF filaments in WT mice (0.16 g pre DHP = 37 ± 5%, 0.16 g post DHP = 44 ± 8%, $P > 0.05$; 0.4 g pre = 64 ± 6%, 0.4 g post = 55 ± 9%, $P > 0.05$; 0.6 g pre = 73 ± 6%, 0.6 g post = 67 ± 7%, $P > 0.05$; 1 g pre = 87 ± 4%, 1 g post = 69 ± 7%, $P > 0.05$; Figure 58). DHP had no impact on the response rate to the 0.16 g, 0.6 g and 1 g vF filaments in *ob/ob* mice (0.16 g pre DHP = 35 ± 7%, 0.16 g post DHP = 36 ± 7%, $P > 0.05$; 0.6 g pre = 76 ± 6%, 0.6 g post = 63 ± 7%, 1 g pre = 87 ± 4%, 1 g post = 78 ± 7%, $P > 0.05$), but DHP did reduce the response rates of *ob/ob* mice to the 0.4 g filament (pre DHP = 66 ± 8%, post DHP = 44 ± 8%, $P < 0.05$; Figure 58). This is consistent with DHP reducing mechanical allodynia, but having no effect on mechanical nociceptive pain.

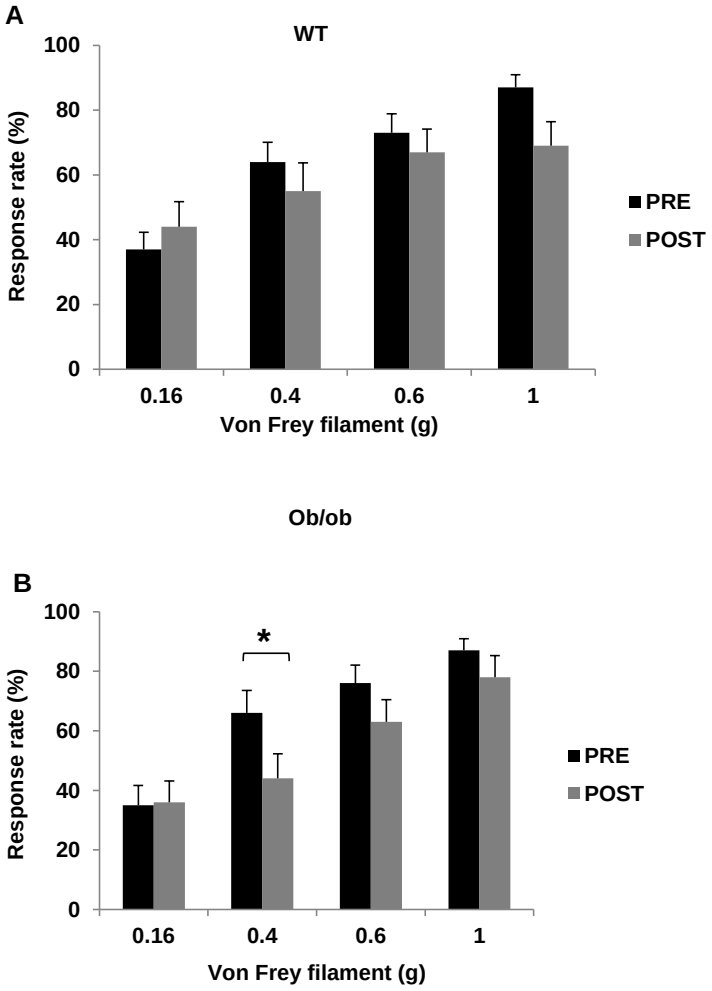

Figure 58 DHP reduces mechanical allodynia in *ob/ob* mice but has no significant effect in WT mice.

(A) Histogram illustrating that DHP (the precursor for allopregnanolone) had no impact on the response to any of the vF filaments in the WT mouse (0.16 g pre DHP = 37 ± 5%, 0.16 g post DHP = 44 ± 8%, $P > 0.05$; 0.4 g pre = 64 ± 6%, 0.4 g post = 55 ± 9%, $P > 0.05$; 0.6 g pre = 73 ± 6%, 0.6 g post = 67 ± 7%, $P > 0.05$; 1 g pre = 87 ± 4%, 1 g post = 69 ± 7%, $P > 0.05$). **(B)** Histogram illustrating that DHP had no impact on the response to the 0.16 g, 0.6 g and 1 g vF filaments in the *ob/ob* mouse (0.16 g pre DHP = 35 ± 7%, 0.16 g post DHP = 36 ± 7%, $P > 0.05$; 0.6 g pre = 76 ± 6%, 0.6 g post = 63 ± 7%, 1 g pre = 87 ± 4%, 1 g post = 78 ± 7%, $P > 0.05$), but DHP did reduce the response of the the *ob/ob* mouse to the 0.4 g filament (pre DHP = 66 ± 8%, post DHP = 44 ± 8%, $P < 0.05$). This is consistent with DHP reducing mechanical allodynia, but having no effect on mechanical nociceptive pain.

In summary, *ob/ob* mice exhibit thermal hypoalgesia, cold allodynia, mechanical allodynia, mechanical hyperalgesia and impaired balance and coordination. In WT mice, the neuroactive steroid ganaxolone is analgesic for thermal and mechanical nociceptive pain at 10 mg/kg but impairs rotarod performance at 30 mg/kg. In *ob/ob* mice, ganaxolone (10 mg/kg) is analgesic for mechanical nociceptive pain but, more interestingly, is also effective in reducing mechanical allodynia and mechanical hyperalgesia. In addition, DHP may also be effective for mechanical allodynia. These findings are consistent with the electrophysiological results described in previous chapters, which demonstrate that there is a reduced endogenous neurosteroid tone in mature *ob/ob* mice. Specifically, $GABA_AR$ mIPSCs from layer 2/3 cortical neurones of *ob/ob* mice have a greater response to neuroactive steroids such as ganaxolone than is observed in WT mice.

Chapter 7: Discussion

As discussed in sections 1.2.6 and 1.3.6 of the Introduction, GABA and the $GABA_AR$ have important roles in neurodevelopment. GABA influences neuronal proliferation, migration, differentiation, synapse maturation and signalling during the maturation process (Ben Ari *et al.,* 2007; Di Cristo, 2007; Owens & Kriegstein, 2002). The whole-cell voltage-clamp technique was used to study the changes to $GABA_AR$-mediated mIPSCs that occur during development in neurones from LII of the spinal cord, the nRT and Layer 2/3 of the cerebral cortex. These mIPSCs were mediated by $GABA_ARs$ and as such they were inhibited by the $GABA_AR$ antagonist bicuculline.

7.1 The decay time of $GABA_AR$ mIPSCs decreases with development at three levels of the pain pathway.

During development (P6-P60) there was a reduction of the mIPSC decay time in all three levels of the pain pathway, which was shown to be particularly dramatic within the first three weeks of life (see Figure 10). It is possible that the rate of development may be staggered with different regions reaching maturity at different ages as has been reported in the spinal cord (Inquimbert *et al.,* 2008), but the data amassed was not extensive enough to make firm conclusions on this issue. In neurones from the nRT and layer 2/3 of the cerebral cortex the peak amplitude, rise time, charge transfer and frequency of $GABA_AR$ mIPSCs were all shown to change with development as well.

In LII neurones of the spinal cord, the peak amplitude, rise time and frequency of $GABA_AR$ mIPSCs did not alter significantly, but the charge transfer *i.e.* the area of the mIPSC, decreased with development. This parameter is influenced by peak amplitude and decay time, it is therefore unsurprising that it changed in parallel with changes in decay time. Subsequent electrophysiological work focused on the mature WT L2/3 cortical neurones to facilitate comparison with neurones from mice with neuropathy caused by type-2 diabetes mellitus (T2DM). Results from these experiments informed the behavioural studies that followed. The

behavioural work translated the electrophysiological findings into measurable anti-nociceptive effects in live mice. The mechanical hypersensitivity of *ob/ob* mice and the increased response to ganaxolone reflected my earlier electrophysiological findings.

The electrophysiological data were obtained specifically from neurones of the pain pathway and made under standardised conditions *i.e.* -60 mV and 35^{o}C (see Figure 10; Brown, 2012; Mitchell *et al.,* 2008; Peden *et al.,* 2008). It is worth noting that the recordings were made at the near physiological temperature of 35^{o}C, while recordings made by other authors are typically made at room temperature (~20 ^{o}C) and mIPSC decay time is highly sensitive to temperature (Collingridge *et al.,* 1984). Interestingly, although recordings made at non-physiological room temperature by other authors may have different individual values for $GABA_AR$ mIPSC decay time, they have also observed a relative reduction of decay time with development in multiple regions of the CNS (Brickley *et al.,* 1996; Draguhn & Heinemann, 1996; Dunning *et al.,* 1999; Keller *et al.,* 2001; 2004; Rajalu *et al.,* 2009; Schlichter *et al.,* 2006; Tia *et al.,* 1996). The reduced decay time may reflect the complex maturation process of neural networks and the greater precision of synaptic transmission and is thought to confer an enhancement of sensory perception, motor coordination and cognitive function (Ben Ari *et al.,* 2007; Cohen *et al.,* 2000; Takahashi, 2005). Changes in the subunit composition of $GABA_ARs$ with development such as α1 subunit expression also account for a significant part of the shorter decay time of $GABA_AR$-mediated synaptic events (Fritchy *et al.,* 1994; Laurie *et al.,* 1992; Okada *et al.,* 2000; Vicini *et al.,* 2001).

Indeed, the subunit composition of $GABA_ARs$ influences their functional properties *i.e.* they may have a higher affinity for GABA and remain in the open configuration for a relatively longer duration, resulting in longer mIPSC decay times (Verdoorn, 1994). In dorsal horn neurones of the spinal cord, $GABA_ARs$ with the α2 and α3 subunits are expressed most frequently, resulting in the characteristically long decay times of $GABA_AR$ mIPSCs (Bohlhalter *et al.,* 1996; Knabl *et al.,* 2008). Neurones of the nRT also express $GABA_ARs$ containing α3 subunits primarily and subsequently these exhibit relatively long $GABA_AR$ mIPSC decay times (Bohlhalter *et al.,* 1996; D'Hulst *et al.,* 2009; Knabl *et al.,* 2008; Peden *et al.,* 2008; Sieghart &

Sperk, 2002). In contrast to the other two regions discussed above, the $GABA_AR$s of layer 2/3 cortical neurones express the $\alpha2$ and $\alpha3$ subunits principally in early life, but during the maturation process the $\alpha1$ subunit becomes predominant (Bosman *et al.,* 2002; Fritschy *et al.,* 1994). This is relevant because pharmacological agents that target the $\alpha2$ and $\alpha3$ subunits preferentially may be useful for treating pathological pain conditions related to a lack of physiological GABAergic inhibition (Knabl *et al.,* 2008). Such pharmacological agents could avoid the undesirable anaesthetic and sedative effects of $GABA_AR$ modulation by 'sparing' $GABA_AR$s within the brain that express the $\alpha1$ subunit that is involved in the mediation of these effects (Knabl *et al.,* 2008; Sieghart & Sperk, 2002). Layer 2/3 cortical neurones that have $GABA_AR$s containing the $\alpha1$ subunit exhibit significantly shorter mIPSC decay times and the difference is clearly apparent in my recordings. Indeed, the decay time of P60-75 $GABA_AR$ mIPSCs is approximately one third of that observed in P6-7 mIPSCs. However, it seems likely that the developmental changes observed at all three levels of the pain pathway may be influenced by multiple factors *i.e.* not just subunit composition (Keller *et al.,* 2004; Peden *et al.,* 2008). In regions of the CNS where the $\alpha1$ subunit is not expressed, such as the dorsal horn of the spinal cord, a reduction in the decay time of $GABA_AR$ mIPSCs is also observed (Keller *et al.,* 2004). Moreover, mice that have been genetically modified to lack the gene responsible for the $\alpha1$ subunit of the $GABA_AR$ exhibit shorter mIPSC decay times with development (Bosman *et al.,* 2002; Peden *et al.,* 2008). A shorter decay time of $GABA_AR$ mIPSCs with maturation is associated with a parallel decrease in the endogenous neurosteroid tone in the CNS (Keller *et al.,* 2004; Grobin & Morrow, 2001; Mellon, 2001). In addition, neurosteroids are implicated in the proliferation, differentiation, survival and activation of neurones within the developing nervous system (Belelli & Lambert, 2005; Do Rego *et al.,* 2009; Mellon, 2007; Mellon *et al.,* 2001). Fluctuations in the endogenous neurosteroid tone of pain pathway neurones may therefore play a significant role in the observed reduction of $GABA_AR$ mIPSC decay time (Keller *et al.,* 2004).

7.2 Region specific properties of pain pathway neurones.

Neurosteroids were the focus of the thesis because they are known to modulate the $GABA_AR$ and therefore potentially enhance neural inhibitory tone, which may be altered in pathological states such as type-1 diabetes mellitus (T1DM; Chen & Pan, 2002). Reduced inhibitory tone is associated with hypersensitivity to pain and neurosteroids may have analgesic effects in inflammatory pain (Chen & Pan, 2002; Poisbeau *et al.,* 2005). Pharmacological blockade of either the $GABA_AR$, or the GlyR, results in hyper-excitability of dorsal horn neurones, which is associated with neuropathic pain symptoms (Charlet *et al.,* 2008; Yaksh, 1989). The LII neurones of the dorsal horn of the spinal cord receive fast synaptic inhibition mediated by GlyRs and $GABA_ARs$ (Chery & De Koninck, 1999; Keller *et al.,* 2001). The organisation of LII is complex, but there are thought to be four main cell-types, including: islet, vertical, radial and central cells (Yasaka *et al.,* 2010). The four different cell types within LII enable this layer to have a critical role in the transmission, integration and modulation of nociceptive signals within the dorsal horn (Poisbeau *et al.,* 2005). However, the heterogeneity of cell types and therefore also the synaptic connections was reflected in the decay time of $GABA_AR$ mIPSCs of LII neurones. This meant that while there was a clear reduction of the decay time of $GABA_AR$ mIPSCs of LII neurones with development, there could also be considerable difference in mIPSC decay time between two neurones from the same slice of spinal tissue and also within an individual neurone. The relatively wide variation in decay time of LII $GABA_AR$ mIPSCs was consistent with the literature (Mitchell *et al.,* 2007) but made it very challenging to assess the effects of pharmacological agents on decay time. In contrast, nRT neurones are more uniform and exhibit relatively homogenous $GABA_AR$ mIPSCs with higher frequency than LII neurones. These properties facilitate the use of pharmacological manipulation in order to assess $GABA_AR$ function with greater precision. For this reason, the initial recordings with γ-CD to investigate the neurosteroid tone were performed in nRT neurones in order to establish the optimum method of administration and to characterise its effects at different stages of development. However, it is not practical to make recordings from the nRT neurones of mice

over the age of P25 due to the high density of axonal projections that obscures visualisation of individual neurones (Cox *et al.,* 1997; Pinault & Deschenes, 1998). Therefore, the nRT was an unsuitable region to study the impact of T2DM in the *ob/ob* mouse, which manifests diabetic neuropathy by the age of ~P60 (Drel *et al.,* 2006; Latham *et al.,* 2009). Layer 2/3 neurones of the cerebral cortex are involved in the pain pathway, exhibit relatively homogenous $GABA_AR$ mIPSCs at high frequency and do not have dense axonal projections. Therefore, cortical neurones provided an opportunity to study $GABA_AR$ mIPSCs of mature WT and diabetic mice and also facilitated the effect of neurosteroid compounds to be studied. However, while cortical neurones were used for the later electrophysiological experiments, the effects of neurosteroids were studied at all three levels of the pain pathway.

7.3 $GABA_ARs$ from pain pathway neurones are sensitive to modulation by neurosteroids.

The neurosteroid allopregnanolone and the closely related synthetic analogue ganaxolone have proven efficacy in enhancing the function of synaptic $GABA_ARs$ in the murine dentate gyrus, ventrobasal thalamus, cerebral cortex and the rat spinal cord (Belelli & Herd 2003; Brown, 2012; Hosie *et al.,* 2006; Mitchell *et al.,* 2007). Importantly, in LII spinal neurones, acutely applied allopregnanolone exerted a significantly greater prolongation of $GABA_AR$ mIPSC decay time at P17-25 than at P8-11. This is consistent with the hypothesis that the neurosteroid tone of LII neurones decreases with development, although an altered neurosteroid-sensitivity of the synaptic $GABA_ARs$ cannot be excluded. P17-25 neurones of the nRT were sensitive to the modulatory effect of ganaxolone, which induced an increase in $GABA_AR$ mIPSC decay time. However, the effect was significantly less than allopregnanolone had in LII spinal neurones of the same developmental age. Both allopregnanolone and ganaxolone were applied acutely to adult layer 2/3 neurones of the cerebral cortex to confirm that the $GABA_AR$ mIPSCs of these neurones were sensitive to these neurosteroid agents. At a concentration of 1 μM both compounds increased the decay time of cortical $GABA_AR$ mIPSCs by a

similarly modest amount, as did ganaxolone (3 μM). This raised the question of whether the synaptic $GABA_A$Rs were in conditions of saturation, or (more likely) whether slice tissue penetration was a major rate-limiting factor. Nevertheless, neurones from all three levels of the pain pathway express $GABA_A$Rs that are sensitive to the effect of neurosteroids.

In order to explore the role that an endogenous neurosteroid tone may have in modulating the $GABA_A$Rs of pain pathway neurones, experiments were performed with γ-CD (a barrel-shaped cyclic oligomer of glucose). γ-CD does not modulate the $GABA_A$R directly, but is able to scavenge lipophilic agents such as neurosteroids and thus prevent them from exerting their effects on the membrane-bound $GABA_A$Rs (Shu *et al.,* 2004, 2007). Keller *et al.,* (2004) proposed that there is a relatively high neurosteroid tone early in development that is significantly diminished with maturation. This hypothesis has been confirmed in VB neurones of the thalamus where γ-CD reduced the decay time of $GABA_A$R mIPSCs at P7-8, but not at P10 or P20-24 (Brown, 2012). In order to confirm the optimal method of administration of γ-CD, I made recordings from P6-7 nRT neurones using three methods of application. 1) γ-CD was presented intracelluarly within the recording electrode, 2) γ-CD was applied *via* the extracellular solution, or 3) γ-CD was included in the pipette, in the extracellular recording solution and in the incubation chamber containing the brain slice preparation prior to recording (*i.e.* 2 hours of preincubation). The intracellular application of γ-CD significantly decreased exponential decay time of synaptic GABAergic events in P6-7 nRT neurones. However, when γ-CD was only present in the extracellular solution for at least 5 minutes, the mIPSC decay (τ_W) was not decreased significantly. Furthermore, when γ-CD was present intracellularly, extracellulary and within the pre-incubation chamber it exerted no more effect than when it was only present intracellularly. This finding was consistent with what has been observed in neurones of the ventrobasal thalamus, which have a reciprocal synaptic arrangement with nRT neurones (see Introduction: section 1.1.7; Brown, 2012).

Subsequent to the experiments at P6-7, recordings were made from nRT neurones aged P9-10 and P17-25. In contrast to the significant effect of pipette-applied γ-CD nRT $GABA_A$R mIPSCs on at P6-7, γ-CD had no effect at P9-10 and P17-25. This

finding is also consistent with data for neurones of the ventrobasal thalamus at similar stages of development (Brown, 2012). Taking this into consideration, the findings in the nRT neurones are consistent with the hypothesis that there is a relatively high neurosteroid tone early in development that is significantly diminished with maturation (Keller *et al.,* 2004).

In parallel with the γ-CD experiments in the nRT and ventrobasal thalamus, the effect of γ-CD has also been investigated in layer 2/3 neurones of the cerebral cortex (Brown, 2012). Previous investigations have revealed that there is also an endogenous neurosteroid tone early in development (P7-P15) that is lost by ~P20 (Brown, 2012). I made recordings with γ-CD at P9-10 and P60-75 to confirm these results. Interestingly, I obtained very similar results at P9-10, but at the much later stage of P60-75 I discovered that a modest endogenous neurosteroid tone appeared to have re-emerged as revealed by the impact of γ-CD at this stage. The change at P60-75 was unexpected and indicates that the loss of neurosteroid tone at P20 may only be a temporary developmental phenomenon. This finding raises the possibility that an endogenous neurosteroid tone may play an active role in the maintenance of GABAergic physiological neural inhibition in adult animals as γ-CD does not influence the $GABA_AR$ directly (Shu *et al.,* 2004, 2007) and will be discussed further below.

7.4 The role of neurosteroids on mature layer 2/3 neurones from the cerebral cortex of WT mice.

My data confirming that there is a neurosteroid tone present in pain pathway neurones is important because the loss of GABAergic spinal descending inhibitory tone is associated with neuropathic hypersensitivity (Munro *et al*., 2009; von Hehn *et al*., 2012; Yaksh, 1989; Zeilhofer, 2008). Consequently, progesterone-derived neurosteroid compounds have been investigated as possible analgesic agents for inflammatory, nociceptive and neuropathic pain (Asiedu *et al.,* 2012; Meyer *et al.,* 2010; 2011; Poisbeau *et al.,* 2005). Although there are numerous causes of

neuropathic pain, T2DM is one of the most common worldwide and therefore of specific societal relevance (Davies *et al.,* 2006; Edwards *et al.,* 2008; Kaplan, 2006). Mouse models for T2DM such as the *ob/ob* and *db/db* mice have been characterised and established as being reliable and spontaneous exhibitors of neuropathy by the age of P60-75, which facilitated their use in my project (Drel *et al.,* 2006, Latham *et al.,* 2009; Sullivan *et al.,* 2006). However, before performing experiments on mice with T2DM, it was necessary to explore the effects of neurosteroids on inhibitory synapses of L2/3 cortical neurones of WT control mice, (refer to Figure 3 for details of the neurosteroid synthesis pathway). Subsequently my work focused solely on L2/3 neurones of the cerebral cortex in order to explore whether these neurones are sensitive to neurosteroids and whether they have the enzymes necessary for endogenous synthesis.

Allopregnanolone and its synthetic analogue ganaxolone exerted a similarly modest prolongation of the decay time of $GABA_AR$ mIPSCs in mature WT L2/3 cortical neurones when applied acutely *via* the extracellular bath. In addition, a three-fold increase in the concentration of acutely applied ganaxolone made no difference to the magnitude of this effect. These results may suggest that such steroids have a limited efficacy at these inhibitory synapses of mature cortical neurons, or alternatively the results are consistent with the concept that drug penetration of the brain slice tissue may be a significant effect-limiting factor (Benkwitz *et al.,* 2007; Gredell *et al.,* 2004). In contrast to the relatively modest prolongation of $GABA_AR$ mIPSCs by acute allopregnanolone described above, a two-hour pre-incubation of the brain slice with allopregnanolone produced a large increase in the decay time of cortical $GABA_AR$ mIPSCs in a concentration-dependant manner.

Acute ganaxolone also had a similar modulatory effect on $GABA_AR$ mIPSCs as allopregnanolone. In common with allopregnanolone, pre-incubation greatly increased the effect of ganaxolone on mIPSC duration, although it was still not as potent in this respect as allopregnanolone. The lower potency of ganaxolone is broadly consistent with previous work in rat brain cortical membranes, which indicated that allopregnanolone was 1.6-fold more potent than ganaxolone in that setting (Carter *et al.,* 1997). Ganaxolone is a synthetic neuroactive steroid with a

very similar structure to allopregnanolone except for the addition of a methyl group at the 3 position of the steroid A-ring. The methyl group, protects the critical 3α- hydroxyl group from metabolism to DHP (Carter *et al.,* 1997). The apparent metabolic stability makes it more effective than allopregnanolone in dentate gyrus neurones, which express the enzyme 3α-HSD (Belelli & Herd, 2003) and enhances its therapeutic potential as it has a long half-life (~20 hours; Luszczki, 2009; Monaghan *et al.,* 1997; Nohria & Giller, 2007). In humans, ganaxolone is metabolised by the hepatic cytochrome enzyme CYP3A4 to 16-OH-GNX and undergoes renal and alimentary elimination (Luszczki, 2009; Nohria & Giller, 2007).

The large difference in the effect of acute application and two-hour incubation of allopregnanolone and ganaxolone is consistent with the slow brain slice penetration time observed with the lipophilic anaesthetics etomidate and propofol (Benkwitz *et al.,* 2007; Gredell *et al.,* 2004). However, the difference in the efficacy between allopregnanolone and ganaxolone incubation treatments raises the question of why the apparently more metabolically stable compound is less effective. The next section considers the effect of the intracellular application of these drugs *via* the recording pipette, which overcomes the issue of brain slice penetration, while minimising the potential for metabolism to occur. This method also explores the potential for autocrine modulation of the $GABA_AR$ by neurosteroid compounds.

Pipette-applied allopregnanolone has previously been investigated in hippocampal neurones without evidence of efficacy. However, the authors had only used nanomolar concentrations of the steroid (Park *et al.,* 2011). In contrast, Akk *et al.,* (2005) were able to confirm that the pipette is a viable method of drug application using the synthetic neuroactive steroid (3α5α,17β)-3-hydroxyandrostane-17-carbonitrile in HEK293 cells. This steroid is membrane-impermeable and was able to modulate the $GABA_AR$ when administered intracellularly. In keeping with the results from Akk *et al.,* (2005), pipette-applied allopregnanolone and ganaxolone induced significant increases in the $GABA_AR$-mediated mIPSC decay time of L2/3 cortical neurones. Interestingly, intracellular allopregnanolone induced a greater prolongation of $GABA_AR$ mIPSC decay time than ganaxolone. Collectively, these

results are consistent with the proposal that allopregnanolone has a greater potency for cortical $GABA_A$Rs than ganaxolone and/or that ganaxolone is converted to allopregnanolone. The effectiveness of intracellular neuroactive steroid administration is also consistent with the reduction of $GABA_A$R mIPSC decay time observed in the presence of intracellular γ-CD. Hence, $GABA_A$Rs may be subject to physiological autocrine modulation by endogenous neurosteroids (Akk *et al.,* 2005; Chisari *et al.,* 2009).

7.5 Intracellularly applied γ-CD reduces the decay time of $GABA_A$R mIPSCs of layer 2/3 cortical neurones in mature WT mice.

In the first Results section, γ-CD was used to reveal the presence of endogenous neurosteroids at different stages of development and at different levels of the pain pathway. The results were consistent with the presence of a relatively high neurosteroid tone in early life that diminishes during development, but reappears in mature cortical neurons, suggesting a physiological role. For example, in inflammatory pain neurosteroid levels may be up-regulated in spinal cord neurones in order to mediate an endogenous analgesic effect (Poisbeau *et al.,* 2005). The CDs were used to investigate the apparent neurosteroid tone in mature L2/3 cortical neurones based on previous experience with these compounds. In order to address the issue that γ-CD itself may be affecting $GABA_A$R function directly, recordings with α-CD and β-CD were made in L2/3 cortical neurones of mature WT mice. The three principle types of CD are the α-CD hexamer, the β-CD heptamer and the γ-CD octomer and they have internal diameters of 5.2 nm, 6.4 nm and 8.3 nm respectively (Cooper *et al.,* 2005; Davis & Brewster, 2004; Ohtani *et al.,* 1989; see section 1.3.3 of the Introduction for more details). My recordings confirmed that the smaller α-CD and β-CD had no impact on $GABA_A$R mIPSCs. This suggests that the size and structure of the CD ring may be critical to its ability to sequester lipophilic compounds such as neurosteroids efficiently and also that γ-CD may not exert a significant direct effect on cortical $GABA_A$Rs or the lipid membrane itself, which is consistent with the findings of Shu *et al.,* (2007).

The apparent removal of neurosteroid tone observed with γ-CD is consistent with the literature (Brown, 2012; Shu *et al.,* 2004; 2007) as was the lack of effect of α-CD (Brown, 2012; Shu *et al.,* 2004). The lack of effect of β-CD on the $GABA_AR$ is consistent with data published by Shu *et al.* (2004). However, it conflicts with conclusions reached by Pytel *et al.* (2006), that β-CD decreased the desensitisation kinetics of the $GABA_AR$ to GABA by direct modulation of the receptor itself within outside-out patches of cultured hippocampal neurones. The mechanism for this direct modulation was uncertain and the findings of Pytel *et al.* (2006) have yet to be replicated. Separately, incubation with ADVASEP-7 (a different isomer of β-CD) was shown to be able to prevent allopregnanolone from augmenting the effect of ethanol incubation in hippocampal neurones within brain slices (Murayama *et al.,* 2006).

The three types of CD discussed above may be relatively non-specific scavengers of lipophilic compounds, which raises the possibility that they could remove other endogenous $GABA_AR$-modulatory compounds. For instance, γ-CD is able to sequester Δ9-tetrahydrocannabinol, but is less effective than both α-CD and β-CD, which are used as vehicles to sequester cannabinoids and deliver them in patients with eye disorders such as glaucoma (Hippalgaonkar *et al.,* 2011; Kearse & Green, 2000; Loftsson & Brewster, 2011; Mannila *et al.,* 2005). In my experiments, pipette-applied α-CD and β-CD had no effect on cortical $GABA_AR$ mIPSC decay time, while γ-CD induced a significant effect. Unpublished data by a former laboratory colleague found that in HEK cells, γ-CD was unable to sequester cannabinoids effectively (Holmgren, Unpublished). Thus the possibility that the γ-CD effect in my experiments is related to the sequestration of endocannabinoids is unlikely. In addition, while γ-CD is able to sequester allopregnanolone, it is not able to sequester the benzodiazepine lorazepam (Shu *et al.,* 2004), making the involvement of any theoretical endogenous benodiazepines also unlikely.

If γ-CD is able to sequester endogenous neurosteroids, then it should also be able to do likewise for exogenously applied neurosteroids. Following brain slice incubation with ganaxolone, or DHP, pipette-applied γ-CD reduced but did not eliminate the effects of the neuroactive steroid, or the neurosteroid precursor. These findings are consistent with the idea that γ-CD is indeed able to sequester

neuroactive steroids in this setting, but that γ-CD may become saturated (Loftsson & Brewster, 1996) and therefore surplus neurosteroid would be able to modulate the $GABA_AR$ (Shu *et al.,* 2004). These findings are also consistent with reports in the literature of γ-CD being able to reverse the effect of allopregnanolone on $GABA_ARs$ of *Xenopus laevis* oocytes (Shu *et al.,* 2004; 2007). In addition, using cultured hippocampal neurones, Shu *et al.,* (2007) found that the intracellular accumulation of the fluorescently-tagged analogue of allopregnanolone, C11-NBD-3α5α could be reduced by the application of γ-CD. The effect of γ-CD was thought to be due to the sequestration of C11-NBD-3α5α from the neuronal membrane. Shu *et al.,* (2007) also reported that γ-CD sequestered other neuroactive steroids such as alphaxolone, pregnane sulphate and 3α5β-THDOC in cultured hippocampal neurones.

7.6 Can mature layer 2/3 cortical neurones synthesise neurosteroids?

My previous experiments have demonstrated that L2/3 cortical $GABA_ARs$ are sensitive to neurosteroids and that there appears to be an endogenous neurosteroid tone. The next part of my study explored the neurosteroid synthesis pathway (Figure 3) in order to test whether L2/3 cortical neurones are able to manufacture progesterone-derived neurosteroids. Two hours of incubation with the neurosteroid precursor progesterone produced a modest prolongation of cortical $GABA_AR$ mIPSC decay time in WT mice that was not concentration-dependent from 1 μM - 50 μM. These data indicate that L2/3 cortical neurones are able to synthesise $GABA_AR$-modulatory neurosteroids when their precursor is administered by brain slice incubation, suggesting that enzymatic function (5α-R and 3α-HSD) is intact. This is consistent with a previous report in spinal dorsal horn neurones from rats (Inquimbert *et al.,* 2008), in which the authors found that incubation of spinal cord slice tissue with progesterone prolonged the decay time of $GABA_AR$ mIPSCs. Interestingly, the authors also demonstrated that co-incubation with the 5α-R inhibitor finasteride prevented the effect of progesterone on $GABA_AR$ mIPSCs but had no impact when administered alone (Inquimbert *et al.,*

2008). Finasteride pre-treatment is also known to be able to prevent the conversion of progesterone into its active neurosteroid metabolites in the hippocampal CA1 neurones of mature mice (Sanna *et al.,* 2004). Inhibition of 5α-R activity also reduces the duration of synaptic GABAergic events in immature neurones of LII of the spinal cord, the ventrobasal thalamus and of the cerebral cortex (Brown, 2012; Inquimbert *et al.,* 2007; Keller *et al.,* 2004; Puia *et al.,* 2003). However, finasteride was ineffective in LII neurones of rats aged > P30 (Keller *et al.,* 2004) and separately in LII neurones aged > P21 (Inquimbert *et al.,* 2008). These reports are consistent with the hypothesis that there is a high endogenous neurosteroid tone in immature animals that has a significant modulatory effect on the $GABA_AR$ (Inquimbert *et al.,* 2008; Keller *et al.,* 2004). Consistent with these reports, in my experiments, finasteride had no effect on $GABA_AR$ mIPSCs, but was able to block the effect of progesterone. However, the lack of effect of finasteride on $GABA_AR$ mIPSCs contrasts to the significant effect of γ-CD described earlier. This difference may be accounted for as follows: γ-CD was pipette-applied directly into the intracellular compartment of the neurone and could thus exert an immediate scavenging effect. Finasteride is able to prevent the conversion of progesterone to more active metabolites, but it would not be able to remove the active neurosteroids that were already present. Further experiments with much longer finasteride incubations could attempt to investigate this matter but it would be challenging to keep the brain tissue from mature animals viable for such a long duration (*i.e.* 6-12 hours). Poisbeau *et al.,* (2005) reported that spinal cord slice incubation with finasteride for more than six hours had no impact on the decay time of $GABA_AR$ mIPSCs in LII neurones of rats aged >P30. However, they also found that it took at least five hours of incubation with finasteride to inhibit the prolongation of $GABA_AR$ mIPSCs associated with carrageenan (seaweed) - induced peripheral inflammation (Poisbeau *et al.,* 2005). Alternatively, finasteride could be injected systemically into live mice at specific times before performing electrophysiological work on their brain tissue to confirm the presence of the endogenous neurosteroid tone by another method. Indeed, Puia *et al.,* (2003) found that a single *in vivo* intraperitoneal injection of the 5α-R inhibitor SKF105111 caused a rapid decrease in the duration of synaptic GABAergic events that lasted more than five hours in the cortical neurones of mice. Intraperitoneal

application of SKF105111 was significantly more effective and occurred sooner than was observed with brain slice incubation treatment (Puia *et al.,* 2003).

The progesterone metabolite DHP is the direct precursor of allopregnanolone, but does not modulate the $GABA_AR$ directly (Belelli & Herd, 2003; Brown, 2012). In keeping with this fact, I found that when DHP was applied acutely either *via* the extracellular bath or *via* the pipette it had no effect on cortical $GABA_AR$ mIPSCs. In contrast, two-hours of incubation with DHP produced a significant, concentration-dependent prolongation of L2/3 cortical $GABA_AR$-mediated mIPSC decay time in WT mice suggesting that 3α-HSD function is both required and also preserved in this setting (Belelli & Lambert, 2005; Mellon *et al.,* 2001; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003). A former laboratory colleague discovered previously that DHP reached its peak effect after two hours, at which time there was a plateau in the modulation of $GABA_AR$ mIPSCs in neurones of the ventrobasal thalamus (Brown, 2012). In addition, the effect of DHP incubation treatment could be reduced by pipette-applied γ-CD in a similar fashion to that of ganaxolone incubation treatment, consistent with the idea that DHP is metabolised to $GABA_AR$ modulatory neurosteroid metabolites.

To further investigate the concept of DHP as a precursor, I investigated the effect of inhibitors of 3-αHSD, including provera and indometacin. Provera inhibits 3α-HSD and therefore prevents the conversion of DHP to allopregnanolone (Belelli & Herd, 2003; Belelli & Lambert, 2005; Sunde *et al.,* 1982). As described in section 1.3.3 of the Introduction, 3α-HSD can also catalyse the reverse reaction of allopregnanolone to DHP, thus inhibition of this enzyme enhances the effect of exogenously applied allopregnanolone in dentate gyrus neurones of rats (Belelli & Herd, 2003). Interestingly, in such dentate gyrus neurones, the synthetic neurosteroid ganaxolone had a greater effect on GABAergic synaptic events than allopregnanolone, which could be inactivated by 3α-HSD (Belelli & Herd, 2003). In my studies, provera inhibited the effect of DHP but not ganaxolone, which was consistent with reports in the literature and also the idea that inhibition of 3α-HSD has no direct effect on the $GABA_AR$ in itself (Belelli & Herd, 2003).

Indometacin has an entirely different molecular structure to provera, but it is also an effective inhibitor of the enzyme 3α-HSD (Askonas *et al.,* 1991; Belelli & Herd, 2003; Belelli & Lambert, 2005; Duax *et al.,* 1978; Hori *et al.,* 2006; Inquimbert *et al.,* 2008; Sunde *et al.,* 1982). Indometacin prevented the effect of DHP on $GABA_AR$ mIPSCs, which was consistent with the effect of provera. However, indometacin also inhibited the effect of ganaxolone incubation treatment on $GABA_AR$ mIPSC duration. These results were unexpected, given that ganaxolone is a closely related synthetic structural analogue of allopregnanolone and interacts directly with the $GABA_AR$ to enhance function (Belelli & Herd, 2003; Belelli & Lambert, 2005; Carter *et al.,* 1997). One possible explanation for these findings would be that indometacin, although having no effect on $GABA_AR$ function *per se* competes for the $GABA_AR$ binding site with ganaxolone, but not allopregnanolone, although given their structural similarity this appears unlikely. Another possible explanation is that ganaxolone could be a precursor for allopregnanone and indometacin is preventing the enzymatic demethylation of ganaxolone.

In order to investigate if indometacin had a direct modulatory effect on the cortical $GABA_ARs$, recordings were made in WT mice after at least two hours of incubation with the drug. Indometacin had no effect on the $GABA_AR$ mIPSC decay time of cortical neurones from either WT or diabetic mice. This contrasts to the modest modulatory effect of provera in WT cortical neurones (see Section 4.12) and also to the previous report of the ability of indometacin (and provera) to prolong the $GABA_AR$ mIPSC decay time of dentate gyrus neurones by inhibiting the inactivation of allopregnanolone (Belelli & Herd, 2003). However in other experiments by the same authors, neither indometacin nor provera prolonged the decay time of $GABA_AR$ mIPSCs of hippocampal CA1 pyramidal neurones (Belelli & Herd, 2003). Indeed, the differential impact of neurosteroids may be related to neurone-specific differences in local steroid metabolism (Belelli & Herd, 2003). My studies also included recordings where indometacin was applied intracellulary *via* the recording pipette. Indometacin had no effect on cortical $GABA_AR$ mIPSCs of L2/3 cortical neurones and when it was co-applied with ganaxolone in the pipette it did not alter the impact of ganaxolone. These findings are inconsistent with the idea

that indometacin could be preventing the effect of ganaxolone incubation treatment by competing for the $GABA_AR$ with ganaxolone.

Interestingly, in humans, indometacin itself is metabolised by hepatic demethylation to inactive metabolites principally by the enzyme CYP2C9, a subtype of the cytochrome P450 family of enzymes (Nakajima *et al.,* 1998). The expression of the CYP2C subfamily of enzymes is variable between mammalian species, but it is expressed in several regions including the CNS of humans and rodents and notably, the cerebral cortex (Gervasini *et al.,* 2001; McFadyen *et al.,* 1998; Miksys & Tyndale, 2002; Oyama *et al.,* 2004). The CYP2C subfamily of enzymes is known to metabolise neurosteroids (McFadyen *et al.,* 1998; Miksys & Tyndale, 2002) and cholesterol is metabolised to pregnenolone by the enzyme CYP450scc (Miksys & Tyndale, 2002; Schumacher *et al.,* 2012). Therefore, it is theoretically possible that indometacin could compete with ganaxolone for the demethylating effects of the CYP2C9 enzyme. This would explain why ganaxolone incubation treatment is less effective than allopregnanolone and inhibited by indometacin but not provera (in contrast to DHP which is inhibited by both drugs). It would also explain why pipette-applied ganaxolone is less effective than allopregnanolone and is not inhibited by indometacin in the pipette. The putative demethylation of ganaxolone within the cortical neurones could be investigated in future experiments using inhibitors and inducers of the CYP2C9 enzyme such as valproic acid and rifampicin respectively (Chen *et al.,* 2004; Chen & Goldstein, 2009; Wen *et al.,* 2001).

My experiments using neurosteroid precursors and enzyme inhibitors indicate that enzymatic function (5α-R and 3α-HSD) and therefore neurosteroidogensis is preserved in mature L2/3 cortical neurones. The effect of neurosteroids on $GABA_AR$ mIPSCs after two-hour incubation is as follows: allopregnanolone > ganaxolone > DHP > progesterone. The differences in effect on $GABA_AR$ mIPSCs decay times is considerable and is consistent with the concept of sequential rate-limiting enzymatic steps between progesterone and allopregnanolone (Mellon *et al.,* 2001; Rupprecht *et al.,* 2010; Schumacher *et al.,* 2012; Stoffel-Wagner, 2003). It could be of physiological benefit for neurones to possess a reservoir of inactive precursors that could be converted to modulate the $GABA_AR$ only when required.

In this way, $GABA_AR$ function could be fine-tuned up or down, rapidly and reversibly in an autocrine manner.

Indeed, γ-CD (but not α-CD or β-CD) is able to sequester neurosteroids when applied intracellularly and is in fact most effective when applied *via* this route (rather than extracellular bath application). In addition, the neuroactive steroids allopregnanolone and ganaxolone (but not DHP) are able to modulate $GABA_AR$ function when applied intracellularly. These findings do not prove that neurosteroids may act in an autocrine manner in cortical neurones, but that is the most likely explanation and is consistent with data published previously by Akk et al., (2005) using a membrane-impermeable steroid. Experiments with isolated neurones in culture could also explore this issue further in order to determine conclusively whether autocrine modulation is occurring. These could involve making electrophysiological recordings before the application of the lowest concentration of γ-CD required to remove the endogenous neurosteroid tone, then continuing to record for prolonged periods of time to allow for *de novo* neurosteroidogenesis. Additionally, precursors and inhibitors could also be employed in these isolated neurones in a similar fashion to my experiments in brain slice tissue.

7.7 The role of neurosteroids on mature layer 2/3 neurones from the cerebral cortex of *ob/ob* and *db/db* mice.

As yet, there are no published reports of the electrophysiological characterisation of synaptic $GABA_AR$ function for T2DM. It was therefore unknown whether neurosteroid tone would be altered in mice with diabetic neuropathy. In inflammatory pain, neurosteroidogenesis is up-regulated to mediate a form of endogenous analgesia by enhancing GABAergic neural inhibition (Poisbeau *et al.,* 2005). The *ob/ob* model of T2DM is particularly useful for the study of neuropathic pain because it develops a more clinically relevant form of diabetes than other models (Cefalu, 2006; Drel *et al.,* 2006; Latham *et al.,* 2009; Lindstrom, 2009;

Neubauer and Kulkarni, 2006; Sullivan *et al.,* 2007). However, it should be noted that no animal model of diabetes fully replicates the human phenotype. *ob/ob* mice are deficient of the hormone leptin and it was considered that leptin itself may modulate $GABA_AR$ function (Solovyova *et al.,* 2009). Therefore, in order to exclude leptin as a potential cause of any changes observed, recordings were made in a second mouse model of T2DM, the *db/db* mouse. As described in the introduction section, the *db/db* mouse is able to synthesise leptin but lacks the leptin receptor. As a result, the *db/db* mouse develops super-morbid obesity, but has a more dramatic diabetic phenotype than the *ob/ob* and also manifests a more severe form of neuropathy at the same age (Bates *et al.,* 2005; Cefalu, 06; Neubauer & Kulkarni, 2006).

I discovered that the $GABA_AR$ mIPSCs from both *ob/ob* and *db/db* mice had significantly shorter decay times than the three WT strains. There are numerous possible reasons for this finding including changes to the sensitivity of the $GABA_AR$ itself or changes in the levels of endogenous modulators such as neurosteroids. In view of the fact that the same changes were present in both models of T2DM it appears that leptin is unlikely to be involved. The *ob/ob* and *db/db* mice have entirely different mutations, which suggest it is also unlikely that there is a unifying intrinsic genetic modification of the $GABA_AR$ itself such as an alteration in subunit composition (Chen *et al.,* 1996; Chung *et al.,* 1996; Zhang *et al.,* 1994). A decrease in the sensitivity of the $GABA_AR$ to allosteric modulators such as neurosteroids is a possible explanation for the shorter $GABA_AR$ mIPSC decay times. However, reduced $GABA_AR$ sensitivity is inconsistent with the exaggerated effect of ganaxolone and DHP in *ob/ob* cortical neurones and the similar effect of allopregnanolone in both WT and diabetic cortical neurones. A possible explanation may be that in *ob/ob* and *db/db* mice there is a reduction in the endogenous neurosteroid tone, which is caused by a common pathological insult associated with T2DM. A reduced neurosteroid tone could result in diminished GABAergic inhibition and subsequently a hypersensitive phenotype as explored in the behavioural experiments. It is possible that other endogenous compounds that modulate the $GABA_AR$ such as cannabinoids or the theoretical endozepines may be

affected by a common mechanism in T2DM; however the selective effects of γ-CD (in comparison to the lack of effect with α-CD and β-CD) make this less likely.

There are also reports in the literature that relatively minor genetic differences between mouse colonies could have significant functional impacts in neurodegenerative conditions (Chandra *et al.,* 2005; LaCroix-Fralish *et al.,* 2009; Wijnvoord *et al.,* 2010). The initial control recordings were carried out in WT mice (Charles River UK Ltd), however, the *ob/ob* and *db/db* mice are only available in the UK from a single source (Harlan Laboratories, UK) and the background strains are different for each type of mouse. The three strains are: C57/BL6, B6.V-*Lep*$^{+}$/OlaHsd and BKS.Cg-*Dock7*^{m}*+/Dock7*^{m}+/OlaHsd respectively. To control for potential differences in strains, recordings were made from the lean WT littermates of both models of T2DM, and there were no differences between the three WT strains.

My experiments with γ-CD indicate the presence of a relatively high neurosteroid tone in early life that diminishes during development, but reappears in mature L2/3 cortical neurones. Recordings were made with γ-CD in *ob/ob* and the *db/db* mice and in the three representative WT strains to explore the putative endogenous neurosteroid tone. Interestingly, γ-CD (but not α-CD and β-CD) reduced the $GABA_AR$ mIPSC decay time to similar baseline values for all five lines of mice. These findings are consistent with the hypothesis that mature WT mice possess an endogenous neurosteroid tone that is reduced in *ob/ob* and the *db/db* mice, but that underlying $GABA_AR$ function and sensitivity are otherwise unchanged in cortical neurones from diabetic mice. Thus, in neuropathic pain associated with diabetes neurosteroid levels may be reduced, which contrasts to inflammatory pain, where neurosteroid levels may be up-regulated in order to mediate an endogenous analgesic effect (Poisbeau *et al.,* 2005). This is important because *ob/ob* mice exhibit hypersensitivity to mechanical stimuli and loss of neuronal inhibition may also be associated with hypersensitivity (Eto *et al.,* 2011).

7.8 The effect of neurosteroids on the decay time of $GABA_AR$ mIPSCs from mature layer 2/3 cortical neurones of *ob/ob* mice.

Interestingly, despite the shorter baseline decay time of $GABA_AR$ mIPSCs from layer 2/3 cortical neurones of *ob/ob* mice, the acute application of allopregnanolone or ganaxolone exerted the same modest effects as they had in the WT mice. In addition, two-hour brain slice incubation with allopregnanolone produced the same dramatic concentration-dependent increase in $GABA_AR$ mIPSC decay time in diabetic mice that it had done in WT mice. In keeping with these findings, pipette-applied allopregnanolone induced the same concentration-dependent increase in $GABA_AR$ mIPSC decay time, in *ob/ob* mice as it had done in WT mice. These results are consistent with the idea that $GABA_AR$ sensitivity to allopregnanolone may be very similar in both WT and diabetic mice because allopregnanolone does not require enzymatic conversion to exert its effects on the $GABA_AR$. They are also consistent with the idea that the shorter decay time of $GABA_AR$ mIPSCs of L2/3 cortical neurones of *ob/ob* mice is due to a reduction in endogenous neurosteroid tone that can be rescued by allopregnanolone. The alternative explanation, *i.e.* that the $GABA_ARs$ of *ob/ob* mice are less sensitive to endogenous allopregnanolone is unlikely because exogenously applied allopregnanolone has the same effect in both WT and *ob/ob* mice.

When ganaxolone was applied acutely *via* the extracellular bath or intracellularly *via* the recording electrode, the drug had the same effect in both *ob/ob* and WT mice, which supports the argument that there is no difference in the sensitivity of the $GABA_AR$ to this neuroactive steroid in diabetic mice. However, these results contrast with the greater impact of ganaxolone incubation treatment observed in *ob/ob* mice compared with WT mice and permits the possibility that ganaxolone may potentially be demethylated to allopregnanolone in order to exert maximal effect on cortical $GABA_ARs$. The idea of ganaxolone as both an agonist and precursor to allopregnanolone would also be consistent with the relatively larger effect of ganaxolone incubation in *ob/ob* mice, which may exhibit an upregulation of key enzymes such as CYP2C and 3α-HSD as indicated by the increased effect of

DHP (the endogenous precursor of allopregnanolone) on *ob/ob* cortical neurones. The inhibitory effect of indometacin on ganaxolone incubation treatment is also consistent with the notion of ganaxolone as an active precursor of allopregnanolone as discussed in the previous chapter. In keeping with the data obtained from WT L2/3 cortical neurones, indometacin had no impact on *ob/ob* cortical neurones when present in the pipette or after two hours of incubation treatment. In addition, when indometacin and ganaxolone were co-applied within the pipette, indometacin had no impact on the ability of ganaxolone to prolong cortical $GABA_AR$ mIPSCs.

7.9 Can mature layer 2/3 cortical neurones from diabetic mice synthesise neurosteroids?

An alternative explanation for the shorter decay time of cortical $GABA_AR$ mIPSCs from diabetic mice relative to the three WT strains is that there is a reduction in neurosteroid synthesis. Indeed, the consistent impact of allopregnanolone in both WT and *ob/ob* mice and the exaggerated impact of ganaxolone incubation treatment in the latter suggest that reduced sensitivity to neurosteroids is an unlikely explanation for the shorter $GABA_AR$ mIPSCs. Therefore it may be due to reduced neurosteroid synthesis in mice with diabetic neuropathy. To test whether *ob/ob* mice are able to synthesise endogenous neurosteroids effectively, recordings were made with the precursors progesterone and DHP (see Figure 3).

At least two hours of incubation with progesterone produced a modest prolongation of cortical $GABA_AR$ mIPSC decay time in *ob/ob* mice that was similar to WT controls and was not concentration-dependent from 1 μM - 50 μM. Additional recordings made at the highest progesterone concentration in *db/db* mice were no different to those of the WT or *ob/ob.* These data are consistent with the idea that L2/3 cortical neurones of T2DM mice are able to synthesise $GABA_AR$-modulatory neurosteroids when their precursor (progesterone) is administered by brain slice incubation. Progesterone induced a similar final prolongation of GABAergic exponential decay time in neurones from the diabetic mice. This

suggests that enzymatic function responsible for the endogenous synthesis of allopregnanolone (5α-R and 3α-HSD; Figure 3) is intact in mature layer 2/3 cortical neurones of the *ob/ob* and *db/db* mice. Indeed, due to the shorter baseline decay time of L2/3 cortical $GABA_AR$ mIPSCs of *ob/ob* and *db/db* mice, progesterone actually had a proportionately greater effect in the diabetic mice than it had done in the WT controls. In keeping with what had been observed in the WT mice, incubation treatment with finasteride (a 5α-R inhibitor) also had no effect on *ob/ob* cortical $GABA_AR$ mIPSCs directly, but was able to block the effect of progesterone in *ob/ob* mice.

Two-hour incubation with DHP (the progesterone metabolite) produced a significant, concentration-dependent prolongation of L2/3 cortical $GABA_AR$-mediated mIPSC decay time in diabetic mice. Interestingly, DHP (the allopregnanolone precursor) produced a greater effect in diabetic mice compared to WT controls despite the shorter baseline $GABA_AR$ decay time in the T2DM cortical $GABA_AR$ mIPSCs. This finding suggests that not only is 3α-HSD enzymatic function preserved in mature mice with T2DM, but it may actually be upregulated. Specifically, an increase in 3α-HSD activity may be a partial compensation for a reduced baseline endogenous neurosteroid tone in diabetic mice. In keeping with what had been observed in WT mice, pipette-applied DHP also had no effect on the cortical $GABA_AR$ mIPSCs of *ob/ob* mice. The lack of effect of DHP is consistent with the DHP's role as a metabolic precursor to the active compound allopregnanolone (Figure 3), *i.e.* there is not enough time for DHP to be metabolised and/or the enzyme 3α-HSD is removed by pipette dialysis.

The underlying mechanism responsible for the reduced neurosteroid tone in the diabetic mice is uncertain, but may be due to mitochondrial dysfunction (Bordet *et al*., 2008; Chowdhury *et al*., 2012; Edwards *et al*., 2008; Fernyhough *et al*., 2010; Vincent *et al*., 2010). It has been postulated that hyperglycaemia (high blood glucose concentration) has several detrimental effects including the excessive donation of electrons to the mitochondrial electron transport chain, which induces an increased production of reactive oxygen species (Chowdhury *et al*., 2012; Fernyhough *et al*., 2010; Vincent *et al*., 2010). The increased availability of electrons may lead to the partial reduction of oxygen to neurotoxic superoxide

radicals (Chowdhury *et al*., 2012; Fernyhough *et al*., 2010). Mitochondrial dysfunction could also account for both mechanical hypersensitivity associated with a reduction in mitochondrial-derived neurosteroids and also for thermal hyposensitivity associated with axonal degeneration (Chowdhury *et al*., 2012; Drel *et al*., 2006; Latham *et al*., 2009; Vincent *et al*., 2010). Further detailed information regarding the mechanisms of mitochondrial dysfunction in diabetic neuropathy has been covered by other authors (Chowdhury *et al*., 2012; Fernyhough *et al*., 2010; Vincent *et al*., 2010). The results from my behavioural experiments characterising the diabetic neuropathy phenotype and the response to the neuroactive steroid ganaxolone are considered below in further detail in the final section of this discussion.

7.10 Overview of electrophysiological data.

Analysis of the kinetics of $GABA_AR$ mIPSCs from mouse neurones in slices of tissue from the spinal cord, nRT and the cerebral cortex reveal that the decay time is reduced with development (from day 6 to 60 postpartum). Neurones from these three levels of the pain pathway are also sensitive to modulation by neurosteroids. The use of γ-CD to sequester endogenous neurosteroids is known to cause faster mIPSC deactivation in cortical neurons during early stages of development implying the presence of a tone of positive allosteric modulation, a phenomenon that declined to negligible levels by age P20 (Brown, 2012). However, I discovered that the endogenous neurosteroid tone reappears by the age of 60 days, suggesting that it may have a physiological role at this age. The effect of γ-CD on the kinetics of mIPSCs recorded from cortical neurons of *ob/ob* mice of a similar age was significantly smaller, implying a deficit in endogenous neurosteroid in these animals. Furthermore, the mIPSCs of *ob/ob* mice were more responsive to the neurosteroid precursors DHP and progesterone. The increased response to the precursors may be due to an upregulation of enzyme activity to compensate for a lower basal neurosteroid tone. However there was no change in the dose-response relationship for allopregnanolone between WT and *ob/ob* mice implying

that the sensitivity of $GABA_A$Rs to neurosteroids remains unchanged even though the *ob/ob* mice start from a lower baseline. Ganaxolone is less effective at cortical $GABA_A$Rs than allopregnanolone, and despite having been developed as a metabolically stable analogue of allopregnanolone, its activity was inhibited by indometacin (but not provera). In fact, ganaxolone behaves more like the allopregnanolone precursor DHP in that it has greater efficacy in *ob/ob* mice than WT mice. It is possible that the demethylation of ganaxolone to allopregnanolone is prevented by indometacin as discussed earlier.

The electrophysiological data suggest that there are deficiencies in GABAergic inhibition associated with a reduced neurosteroid tone in *ob/ob* mice. Therefore I examined whether these deficits contributed to aberrant nociception in *ob/ob* mice, such as allodynia and hyperalgesia. The next section will discuss the results of behavioural experiments in WT and *ob/ob* mice, with a focus on ganaxolone, which is considered to have greater bioavailability than allopregnanolone (Carter *et al*., 1997; Gee *et al*., 1995) using tests of thermal and mechanical nociception.

7.11 The impact of neuropathy on behavioural measures of hypersensitivity and the response to neuroactive steroids.

Diabetes is one of the principle causes of neuropathic pain in the human population and, at present, the available therapies are often ineffective (Bouhassira *et al*., 2008; Dworkin *et al*., 2007; Harstall & Ospina, 2003). Nociceptive (or physiological) pain has a protective role, alerting the individual to the presence of a harmful situation from which they should withdraw (Caterina *et al.,* 2005; Costigan *et al.,* 2009; Woolf & Salter, 2000). In contrast, neuropathic pain results from a dysfunctional or damaged nervous system and may be associated with pathological hypersensitivity of the peripheral or central nervous system (Fischer & Waxman, 2010; Harvey & Dickenson, 2008; Merskey & Bogduk, 1994, Woolf & Salter, 2000). Hyperalgesia describes an exaggerated response to a painful stimulus, while allodynia describes an unpleasant or painful response associated with an innocuous stimulus (Merskey & Bogduk, 1994). Of specific relevance,

hypersensitivity of the spinothalamic tract has been reported in animal models of T1DM (Chen *et al*., 2001, Pertovaara *et al*., 2001).

$GABA_AR$s are the major inhibitory receptors in the mammalian nervous system and a reduction of their function is associated with allodynia and hyperalgesia (Munro *et al*., 2009; von Hehn *et al*., 2012; Yaksh, 1989; Zeilhofer, 2008). Ligands that specifically target the benzodiazepine binding site on $GABA_AR$s containing either $\alpha2$ or $\alpha3$ subunits are effective against inflammatory and neuropathic pain without side effects such as sedation, tolerance and ataxia that are associated with anxiolytic benzodiazepines (Knabl *et al*., 2008). In addition, Poisbeau *et al.,* (2005) discovered an increase in neurosteroid production by spinal neurones associated with peripheral inflammatory pain, providing compensatory analgesia. My electrophysiological experiments revealed that diabetic mice had a reduced endogenous neurosteroid tone, but that the neuroactive steroid ganaxolone had an exaggerated effect on cortical $GABA_AR$ mIPSCs. A previous report using the tail flick test found that ganaxolone administered *via* the spinal (or intrathecal) route reduced thermal nociception (Asiedu *et al.,* 2012). In addition, systemic allopregnanolone, DHP and progesterone have had promising effects in rodents for the prevention of chemotherapy-induced neuropathic pain (Meyer *et al*., 2010; 2011).

Animal models are essential for the effective study of neuropathic diseases before clinical studies can be justified (Mogil *et al.,* 2010). However, this is particularly true for diabetic neuropathy because the efficacy of interventions may be studied over weeks or months rather than decades as is the case in human studies (Cefalu, 2006; Kaplan & Wagner, 2006; Neubauer and Kulkarni, 2006; Rees & Alcolado, 2005). Specifically, if it takes twenty years for significant diabetic neuropathy to develop after diagnosis, then it could take a relatively similar length to determine (with a reasonable degree of confidence) if a novel drug is effective at preventing or reducing neuropathy. This contrasts with mouse models in which it can take two months for diabetic neuropathy to develop.

No single animal model fully replicates diabetes in humans and all models have advantages and disadvantages (Cefalu, 2006; Kaplan and Wagner, 2006; Mathis *et*

al., 2001; Sullivan *et al.,* 2007). The *ob/ob* mouse was chosen for study because it develops a predictable neuropathic phenotype by 8 weeks of age, which is comparable to that observed in humans with T2DM (Drel *et al.,* 2006; Latham *et al.,* 2009; Lindstrom, 2009; Vareniuk *et al.,* 2007). Indeed, peripheral neuropathy and associated hypersensitivity have previously been characterized in *ob/ob* mice (Drel *et al.,* 2006; Latham *et al.,* 2009). The first behavioural experiments that I carried out on *ob/ob* mice were to confirm the reported phenotype; these tests included the rotarod, the von Frey (vF) filaments and the tail flick test. In a manner consistent with the literature, the *ob/ob* mice exhibited super-morbid obesity and a dramatically impaired performance on the rotarod test, which assesses sensorimotor coordination (Mayers *et al.,* 2009). This is consistent with the reduced motor and sensory nerve conduction velocities of *ob/ob* mice, which impairs their balance and coordination (Drel *et al.,* 2006; Vareniuk *et al.,* 2007).

The *ob/ob* mice also exhibited significantly longer tail flick withdrawal latencies at 48 ^{o}C and 50 ^{o}C than WT controls. These findings are consistent with the phenomenon of thermal hypoalgesia measured by paw withdrawal latency in *ob/ob* mice reported by Drel *et al.,* (2006), but conflict with the thermal hyperalgesia reported by Latham *et al.,* (2009). Latham *et al.,* (2009) raise numerous potential reasons for the discrepancy in findings, which include: methodological differences in testing, internal biological variability and different environmental, nutritional and housing conditions. In addition, Latham *et al.,* (2009) focused on female *ob/ob* mice while the sex of the mice in the paper by Drel *et al.,* (2006) is unreported. All the *ob/ob* mice in my behavioural and electrophysiological studies were male, but it would be presumptive to infer gender as the explanation for the difference. Of note, the neuroprotection of allopregnanolone in a mouse model of global cerebral ischaemia was greater in female mice than in male mice (Kelley *et al.,* 2011). However, certain strains of WT mice that are available commercially have α-synuclein deficiency, which can affect the expression of neurodegenerative conditions and raises the possibility that there may be additional genetic differences, which could contribute to distinct pain phenotypes (Chandra *et al.,* 2005; Specht & Schoepfer, 2001). While it is important to be aware of this issue, detailed analysis is beyond the scope of this

discussion and this is an issue for the scientific community as a whole, rather than one of specific relevance to my electrophysiological and behavioural data.

The results of my study demonstrate that the tail flick test was not suitable for investigating potential analgesic or anti-hyperalgesic effects of drugs such as neurosteroids in *ob/ob* mice. However, my data indicated that 50 ^{o}C was an appropriate temperature to test for noxious heat and to determine the analgesic potential of specific drugs in WT mice. It is arguable that the impaired performance of *ob/ob* mice on the rotarod and the prolonged tail flick latency time could potentially be due to a sensorimotor deficit rather than thermal hypoalgesia *per se*. However, the exaggerated response of the *ob/ob* mice to topical acetone and von Frey filaments (discussed below) suggests that a mixed picture of altered sensitivity to peripheral noxious stimuli is present.

7.12 Ganaxolone impairs thermal nociception in WT mice.

Neurosteroids such as allopregnanolone, DHP and progesterone have analgesic effects in various experimental models (Poisbeau *et al.,* 2005, Charlet *et al.,* 2008, Meyer *et al.,* 2008; 2010; 2011). In addition, the spinal (intrathecal) administration of ganaxolone induces analgesia assessed using the tail flick test (52^{o}C; Asiedu *et al.,* 2012). Also using the tail flick assay (50 ^{o}C), I discovered that the systemic (intraperitoneal) administration of ganaxolone induced a dose-dependent analgesic effect in WT mice. The route of administration is important because in clinical practice, spinal injections are performed within an operating theatre by a trained medical practitioner and carry a small risk of permanent paralysis (Aromaa *et al.,* 1997). As a result, spinal drug delivery is reserved for specific indications such as invasive surgical procedures in humans. In addition, drugs administered directly into the spinal fluid such as opioids may be more than 100 times more efficacious than the same drug delivered systemically (Rathmell *et al.,* 2005). Therefore promising findings with intrathecal administration may not translate to clinically useful therapies due to systemic side effects. During the tail flick experiments the highest dose of ganaxolone appeared to sedate the mice,

which raised the question of whether increased withdrawal latency was related to sensorimotor impairment. Subsequently, the rotarod test was used to explore this potentially confounding issue further. The highest dose of ganaxolone caused a significant impairment of rotarod performance, but the lower doses had no effect. These data suggest that ganaxolone exhibits an analgesic effect at a dose of 10 mg/kg but only impairs rotarod performance in WT mice at higher doses such as 30 mg/kg. The dose-dependent effect on rotarod performance is consistent with the literature (Carter *et al.,* 1997) and indicates that ganaxolone (10 mg/kg) may be effective for thermal nociceptive pain, rather than merely impairing sensorimotor function.

7.13 *ob/ob* mice exhibit cold allodynia.

The sensing of a wide range of environmental temperatures is a useful physiological process that confers survival advantages in many species. Indeed, adapting to a cold external environment can be as important as withdrawing from a burning hot stimulus. The thermal gated ion channels TRPM8 and TRPA1 are important in the generation of an afferent neural impulse in response to a noxious cold stimulus. Increased sensitivity to cold stimuli is a common symptom associated with neural injury, and although the underlying mechanisms responsible for this phenomenon are poorly understood, it may be related to increased expression of TRPM8 and TRPA1 channels (Belmonte *et al.,* 2009; Knowlton *et al.,* 2010). Conversely, knockout of TRPM8 in mice with a peripheral neuropathic injury results in a significantly reduced response to cold stimuli such as topical acetone (Belmonte *et al.,* 2009). When neural hypersensitivity is present, the cold sensation associated with topical acetone may induce a temporary noxious response. Acetone has therefore also been used to elicit cold allodynia in animals with chemotherapy-induced neuropathic hypersensitivity (Flatters & Bennett, 2004, Gauchan *et al.,* 2009, Meyer *et al.,* 2011). In my study, topical acetone elicited a greater response in *ob/ob* mice than it did in WT mice, which was consistent with cold allodynia in the former. However, it became

apparent that after the initial baseline response to acetone, *ob/ob* mice appeared to acclimate to the stimulus. The partial loss of response with repeated measures meant that the test was not an appropriate way of investigating potential analgesic or anti-allodynic effects of drugs such as neurosteroids. Previous studies in which acetone was used to assess cold allodynia differed from my study in that they utilised WT mice with acute chemotherapy-induced neuropathic pain and were conducted over several weeks (Flatters & Bennett, 2004, Gauchan *et al.,* 2009, Meyer *et al.,* 2011). This is relevant because differences in the mechanism responsible for inducing neuropathic pain may result in a significantly different neuropathic phenotype, *i.e.* the balance between loss of sensation and hypersensitivity may be altered. Indeed, the motor and sensory nerve conduction velocities of the *ob/ob* mouse are significantly impaired (Drel *et al.,* 2006). While the prolonged tail withdrawal could be explained by a slower nerve conduction velocity, cold allodynia (and mechanical hypersensitivity) infers that there is a change in nociception rather than merely an impairment of conduction.

7.14 *ob/ob* mice exhibit mechanical hypersensitivity in comparison to WT mice.

Meyer *et al.,* (2011) described a protocol for the use of vF filaments to investigate allodynia, hyperalgesia and nociceptive pain in relation to specific mechanical stimuli in rats. I developed a modified version of their approach to investigate mechanical hypersensitivity and nociception in WT and *ob/ob* mice. This involved choosing four vF filaments that would induce a response on a predictable number of occasions. The 0.16 g and 0.4 g vF filaments elicit withdrawal responses on 25% and 40% of occasions respectively in the WT mouse and are considered to be mild stimuli. If the *ob/ob* mouse has an exaggerated response to these filaments this would be consistent with allodynia (Merskey & Bogduk, 1994). The 0.6 g vF filament elicits a response on over 60% of occasions in the WT mouse, therefore it is considered a painful response and if the *ob/ob* mouse had an exaggerated response to this filament it would be consistent with hyperalgesia (Merskey &

Bogduk, 1994). The 1 g vF filament elicits a withdrawal response on approximately 90% of occasions, therefore it is considered to be a clear test of mechanical nociceptive pain in the WT mouse.

The *ob/ob* mice exhibited an exaggerated (but reproducible) response to the 0.16 g, 0.4 g and 0.6 g vF filaments which was consistent with the presence of mechanical allodynia (0.16 g and 0.4 g) and mechanical hyperalgesia (0.6 g). Previous reports in the literature have used different methods or equipment for the characterisation of mechanical hypersensitivity, but the results of my study are consistent with their findings (Drel *et al.,* 2006; Latham *et al.,* 2009). These data suggest that the vF filaments are a useful method of investigating the potential analgesic or anti-hyperalgesic effects of drugs such as neurosteroids in *ob/ob* mice.

7.15 Ganaxolone reduces mechanical hypersensitivity in *ob/ob* mice and reduces mechanical nociceptive pain in both WT and *ob/ob* mice.

In electrophysiological experiments using cortical slices from WT mice, ganaxolone and DHP both induced a marked prolongation of $GABA_AR$ mIPSC decay time, although the response to ganaxolone was approximately seven-fold greater than that of DHP. Using the vF filaments, the systemic administration of ganaxolone (at 10 mg/Kg, a dose that did not alter rotarod performance) proved to be analgesic for mechanical nociceptive pain in both *ob/ob* and WT mice. In contrast, DHP (the precursor to allopregnanolone) was ineffective for mechanical nociceptive pain at the same dose. It is not possible to translate electrophysiological findings directly into behavioural effects, but, taken as a whole, the data are consistent with the idea that ganaxolone incubation treatment ultimately has a more potent effect than that of DHP and for this reason potentially has greater anti-nociceptive effects in behavioural tests.

However, the most interesting finding of the behavioural work was that ganaxolone significantly reduced the mechanical allodynia and hyperalgesia manifested by the *ob/ob* mice (DHP was also partially effective for mechanical

allodynia, but not hyperalgesia). These findings are important because, in the clinical setting, effective treatments for various forms of nociceptive pain already exist, such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), opioids and regional anaesthesia for surgical procedures. In contrast, the current therapeutic options for neuropathic pain are very limited, therefore patients with diabetic neuropathy may suffer from intractable pain (Bouhassira *et al.,* 2008; Dworkin *et al.,* 2007). The effectiveness of a single dose of the neuroactive steroid ganaxolone for diabetic neuropathic pain in *ob/ob* mice is a novel discovery, but it is also consistent with the literature. Chronic administration of progesterone, DHP or allopregnanolone has been shown to prevent the development of neuropathic pain in animals associated with chemotherapeutic drugs (Meyer *et al*., 2010; 2011). Chemotherapy is usually a short-lived insult to the nervous system, while diabetes mellitus is a relentless lifelong condition for which there is currently no cure. Therefore, the potential impact of a new therapeutic agent could be far greater for painful diabetic neuropathy than for many other neuropathic conditions. My results indicate that ganaxolone should receive further consideration as a potential treatment for allodynia and hyperalgesia in humans with diabetes. While it was not possible to establish whether or not ganaxolone induced sensorimotor impairment in *ob/ob* mice due to their existing sensorimotor deficits, WT mice exhibited analgesia to thermal and mechanical nociceptive pain at a dose that did not impair rotarod function. The apparent 'therapeutic window' suggests that sedation or sensorimotor impairment is an unlikely reason for the observed effects of ganaxolone at the dose of 10 mg/kg. *ob/ob* mice exhibited the same analgesic effect of ganaxolone to mechanical nociceptive pain but, more importantly, the drug also reduced mechanical allodynia and hyperalgesia. To support the idea that ganaxolone is effective at reducing hypersensitivity in *ob/ob* mice, the drug did not affect the response of WT mice to non-painful stimuli.

Humans with painful neuropathy commonly exhibit a mixed picture of sensory loss and hypersensitivity such as allodynia and hyperalgesia that may occur simultaneously in the same anatomical location (Costigan *et al*., 2009; Kehlet *et al*., 2006; Merskey & Bogduk, 1994, Woolf & Salter, 2000). The behavioural results for

the *ob/ob* mice are consistent with what may be observed in humans and therefore are clinically relevant. However, there are significant limitations of these studies, which must be considered. Humans complain of pain that may be related to a specific stimulus or, as is often the case with neuropathic pain, pain may occur spontaneously in the absence of an identifiable cause (Costigan *et al*., 2009; Kehlet *et al*., 2006; Merskey & Bogduk, 1994). Mice are not able to communicate the presence or severity of pain directly and therefore studies have to rely on behavioural observations as a proxy measure of pain. This is important because in humans with chronic neuropathic pain there may be no outward manifestation of the pain experience and the degree of pain-related behaviour may not correlate well with the perceived intensity of the pain (Wall *et al.,* 2006). Furthermore, it is common practice in clinical trials of analgesic compounds to use a verbal or written rating scale to record the severity of the pain experienced (Wall *et al.,* 2006). For example, the subject is asked to assign their pain a severity score out of ten, with zero being no pain and ten being the worst pain imaginable (Macintyre & Schug, 2007). Clearly this is an unavoidably different end-point from my study, but in future, it is likely to become common practice for more objective measures of nociception to be employed clinically, such as mechanical and thermal thresholds (Salengros *et al.,* 2010).

In addition, it must be understood that the experience of pain in humans is a highly subjective one that is hard to define. It is not merely the reflex withdrawal of the hand from the fire, it may be considered as an emotional state analogous to happiness, anxiety or depression that is highly context specific (Wall *et al.,* 2006). For instance, on the battlefield or sports field, participants may continue to play after sustaining an injury that would normally be associated with severe pain (Beecher, 1946; Wall *et al.,* 2006). In contrast, the same people may later become patients attending the chronic pain clinic and complain of disabling symptoms that occur in the absence of tissue damage or in some cases any identifiable stimuli whatsoever. Attendees may often have significant emotional and psychological components to their symptomatology (Tunks *et al.,* 2008; Wall *et al.,* 2006). For this reason, chronic pain management is typically carried out by a multidisciplinary team, which includes psychologists as well as doctors, nurses and

physiotherapists in order to address the multidimensional needs of an individual patient (Kehlet *et al.,* 2006; Wall *et al.,* 2006). In order to identify and quantify these issues, standardised multidimensional tools such as the McGill pain questionnaire are used to facilitate the implementation of targeted therapeutic interventions (Melzack, 1987). Modified versions of questionnaires such as this are also used increasingly within clinical trials, but unfortunately this type of analysis is not applicable to animal models.

Another significant issue worthy of discussion is the nature of the mechanical and thermal stimuli employed within my studies. For ethical reasons, the types of stimuli chosen were considered to induce discomfort, but not to cause harm to the animal. This creates a somewhat artificial situation, *i.e.* in clinical trials of postoperative pain, human subjects have typically experienced a surgical incision that would be considered to be of much greater severity than the mice were subjected to in my studies. This means that my study has tested the efficacy of ganaxolone on nociception and hypersensitivity rather than on 'pain' as a human may experience it. While this is a significant issue, it is a problem for science as a whole to address *via* translational collaborations rather than something that is unique to my study (Mogil, 2009). Given that humans are considered to be the most intellectually developed animals on earth and therefore also the most self-aware, perhaps a pain experience that is truly reflective may never be replicated in animal models. My study used standardised techniques that are in common scientific practice and therefore considered to be useful and quantifiable proxies for nociception. These techniques may be considered as a pragmatic compromise given time, resources and technologies available currently. Putting aside financial considerations, another complementary modality to examine activity of neurones located within the pain pathway and so-called pain matrix such as functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) could also have been employed (Tracey, 2008; Wartolowska & Tracey, 2009). Activation of structures including the thalamus, somatosensory cortex and cingulate cortex has been observed in both rodent and humans studies using PET and fMRI techniques (Knabl *et al.,* 2008; Thompson & Bushnell, 2012; Tracey, 2008; Wartolowska & Tracey, 2009). Imaging in animal models facilitates the

performance of experiments that would not be possible in humans for ethical and practical reasons (Thompson & Bushnell, 2012; Wartolowska & Tracey, 2009). Specifically, the central actions of potential novel analgesic agents can be investigated to examine whether they may be suitable to put forward for clinical trials in patients with neuropathic pain (Knabl *et al.,* 2008; Thompson & Bushnell, 2012; Wartolowska & Tracey, 2009).

Some of the major causes of severe neuropathic pain in humans include amputation, mastectomy, postherpetic neuralgia, thoracic surgery, trigeminal neuralgia and diabetes mellitus. The accurate replication of these conditions in animals in order to facilitate the development of effective clinical treatments poses significant practical and ethical challenges. It is hoped that a compound that was effective for painful diabetic neuropathy may also be effective for other types of neuropathic pain, but this is by no means certain (Dworkin *et al.,* 2007). This issue could be addressed in part by attempting to replicate the behavioural (and electrophysiological) results in other models of neuropathy. It could also be studied in other species and perhaps through the parallel use of techniques such as fMRI and also mass spectroscopy of neural tissue. Other animal models of neuropathy that have been used elsewhere include streptozotocin-induced T1DM, spared nerve injury, sciatic nerve ligation, chemotherapy-induced neuropathy and postherpetic neuralgia (Mogil, 2009). Clearly, replication of the data before attempting to translate the findings into clinical trials would require a considerable amount of work, which is beyond the scope of this thesis. However, fMRI has already been used to characterise the changes in central processing associated with chronic cyclical dysmenorrhoea in women who were also more sensitive to thermal stimuli than healthy controls (Vincent *et al.,* 2011). Dysmenorrhoea is associated with fluctuations in the circulating levels of steroid hormones including oestradiol, testosterone, progesterone and other neurosteroids (Maguire & Mody, 2009; Vincent *et al.,* 2011). Changes in steroid hormone levels during the menstrual cycle are associated with changes in neuronal excitability, which is thought to be mediated *via* their impact on $GABA_A$Rs (Lovick, 2008; Maguire & Mody, 2009).

The results of my studies suggest that a single dose of ganaxolone is effective at reducing mechanical hypersensitivity and nociceptive pain *via* the modulation of $GABA_A$Rs. However, this finding is only the first step in characterising the efficacy of the drug for the treatment of neuropathic pain. Diabetes mellitus is currently an incurable and life-long condition in humans that is associated with progressive multisystem degeneration, including the nervous system (Davies *et al.,* 2006). Careful optimisation of glycaemic control slows the progression of the disease, but is not able to prevent it entirely (Edwards *et al.,* 2008). Patients with diabetes may experience chronic neuropathic hypersensitivity and therefore any potential therapy would need to be administered chronically. Analgesic drugs such as paracetamol and NSAIDs maintain their (limited) efficacy for prolonged periods of time, while opioid drugs are associated with the development of tolerance as well as other serious side effects (Ballantyne & Mao, 2003; Raghavan *et al.,* 2011). A single dose of ganaxolone is promising for neuropathic hypersensitivity, but it is uncertain if this compound would continue to be effective if administered indefinitely or whether tolerance would develop. In order to explore this further, ganaxolone could be administered regularly to *ob/ob* mice over a period of weeks or months, with serial measurements of nociception. Such a protocol could be the first step to test whether ganaxolone was able to prevent the development of hypersensitivity and if tolerance to its effect developed over time. My electrophysiological data suggest that ganaxolone (and allopregnanolone) may have significantly greater potency at the $GABA_A$R than the neurosteroid precursors progesterone and DHP. In view of the fact that these compounds may potentially be active at other targets (especially in high concentrations) it seems rational to focus on the compounds with the greatest potency. Indeed, side effects are often the rate-limiting factor in the clinical efficacy of commonly used analgesics (Dworkin *et al.,* 2007; Wall *et al.,* 2006). In addition, in my behavioural work, I found that the neurosteroid precursor DHP was significantly less effective than ganaxolone for the reduction of mechanical nociception and hypersensitivity.

The electrophysiological work that I carried out confirmed the concentration-dependent effect of neuroactive steroids on $GABA_A$R function by reductionist methodology, while the behavioural work attempted to determine whether these

findings could be translated into a relevant effect in live animals. In the former technique the $GABA_AR$ was isolated using antagonists to block other receptors, while in the latter technique, no such antagonists were used. (Antagonists for glycine and sodium channels (strychnine and TTX respectively) are lethal toxins *in vivo*). Ganaxolone was designed specifically to be a high-affinity $GABA_AR$ modulator and radioligand binding techniques have demonstrated that it has negligible affinity for a large number of non-target receptors, including: excitatory amino acid, inhibitory amino acid, peptide, monoamine and cytosolic steroid receptors (Carter *et al.,* 1997). This suggests that the effect of ganaxolone on nociception and hypersensitivity may be associated with its modulation of $GABA_AR$ function. It was not possible to correlate the *in vitro* and *in vivo* brain tissue concentrations of ganaxolone directly to determine if they were analogous, but in the former the drug had a concentration-dependent effect, while in the latter it had a dose-dependent effect. Therefore my data identify ganaxolone and possibly other neurosteroidal compounds as potential therapeutic agents which could reduce painful hypersensitivity by the selective targeting of the $GABA_AR$. The results of my behavioural experiments are promising, but must be considered in the context of all the issues discussed above. The inherent simplifications and limitations of my studies make it difficult to extrapolate with any certainty that ganaxolone could be a useful clinical agent for allodynia or hyperalgesia. However, my studies are a pragmatic and promising first look at the potential effectiveness of neuroactive steroids for painful diabetic neuropathy.

Chapter 8: Conclusions

This study used a combination of electrophysiological and behavioural techniques in order to investigate the potential for neurosteroids to exert an analgesic effect *via* modulation of the $GABA_AR$. The approach consisted of the characterisation of pain pathway neurones from both WT and diabetic mice followed by the *in vitro* and *in vivo* application of neurosteroidal compounds. The study confirmed that the decay time of $GABA_AR$ mIPSCs of neurones from three levels of the pain pathway decreases with development; lamina II of the spinal cord, the nRT of the prethalamus and layer 2/3 of the cerebral cortex. The neurodevelopmental process occurs in parallel but may take place earlier in the more primitive regions of the CNS. In addition, $GABA_ARs$ of neurones from all three levels of the pain pathway are sensitive to neurosteroids. The acute application of allopregnanolone prolonged the decay time of $GABA_AR$ mIPSCs more effectively in spinal LII neurones from older mice consistent with the idea that immature mice already have a high endogenous neurosteroid tone. γ-CD within the recording pipette significantly reduced the decay time of $GABA_AR$ mIPSCs of nRT neurones of mice aged P6, but not P9-10 or P17-25. This finding was also consistent with the hypothesis that immature mice have a high endogenous neurosteroid tone, which decreases with development. Surprisingly, γ-CD reduced the decay time of cortical $GABA_AR$ mIPSCs at P60-75, suggesting that the endogenous neurosteroid tone, which disappears around P20, re-emerges in adult mice.

Neurosteroid tone appears to be reduced in diabetic mice. As such, the $GABA_AR$ mIPSCs of cortical layer 2/3 neurones of two mouse models of T2DM (*ob/ob* and *db/db*) have shorter decay times than three strains of WT mice. γ-CD reduced the decay time of cortical $GABA_AR$ mIPSCs to the same baseline value in T2DM and WT mice irrespective of strain. In contrast, α-CD and β-CD had no effect. Cortical $GABA_ARs$ of WT and *ob/ob* mice were modestly sensitive to the neurosteroids allopregnanolone and ganaxolone, but not the precursor DHP. Incubation treatment with neurosteroids and their precursors produced more dramatic effects on $GABA_AR$ mIPSCs than acute treatment had done. This may have been partly related to long penetration times of these lipophilic agents in brain slice tissue. Neurosteroids from sequential stages of the synthesis pathway induced

significantly different degrees of modulation of $GABA_AR$ mIPSCs; allopregnanolone > DHP > progesterone. In addition, ganaxolone and DHP (but not allopregnanolone) had an exaggerated effect on cortical $GABA_ARs$ of mice with T2DM in comparison to WT controls. Of relevance, the neurosteroid synthesis pathway relies on the function of key enzymes including 5α-R and 3α-HSD. Inhibition of these enzymes prevented the effect of the precursors, progesterone and DHP respectively. The 3α-HSD inhibitor indometacin had no impact on allopregnanolone incubation treatment itself, but was able to inhibit the neuroactive steroid ganaxolone and the precursor DHP. Incubation with the 3α-HSD inhibitor provera itself induced a modest increase in $GABA_AR$ mIPSC decay time, while indometacin and the 5α-R inhibitor finasteride had no effect. These drugs may inhibit the endogenous production of more neurosteroids, but they are unable to remove neurosteroids that are already *in situ*. In contrast, the neurosteroid scavenger γ-CD removed the endogenous neurosteroid tone and was also able to reduce the effect of ganaxolone and DHP incubation treatments.

Pipette-applied neurosteroids allopregnanolone and ganaxolone (but not DHP) were also able to prolong the decay time of cortical $GABA_AR$ mIPSCs in a differential manner. Allopregnanolone induced a greater effect than ganaxolone on $GABA_AR$ mIPSC decay time, but there was no difference in the response to the respective drugs between the WT and *ob/ob* mice. These findings suggest that neurosteroids are able to modulate the $GABA_AR$ from the intracellular compartment and that the sensitivity of the $GABA_AR$ is the same for WT and *ob/ob* mice. In addition, when indometacin was co-applied in the pipette with ganaxolone it had no impact on the efficacy of ganaxolone. This observation is inconsistent with the notion that indometacin competes with ganaxolone for its binding of the $GABA_AR$.

Ob/ob mice developed obesity and T2DM on a normal diet, which led to painful neuropathy by the age of P60-75. At this age they exhibited sensorimotor impairment, thermal hypoalgesia, cold allodynia, mechanical allodynia and mechanical hyperalgesia. In WT mice the neuroactive steroid ganaxolone impaired sensorimotor function at 30 mg/kg, but the lower dose of 10 mg/kg did not, and was analgesic for thermal and mechanical nociceptive pain. The effect of ganaxolone on thermal nociception and sensorimotor impairment could not be tested on *ob/ob* mice due to their pre-existing deficits. However, ganaxolone significantly reduced mechanical allodynia and mechanical hyperalgesia in *ob/ob* mice. These findings translate the electrophysiological results into behavioural effects *in vivo* and suggest that $GABA_AR$-modulatory neurosteroidal drugs have analgesic properties for nociceptive and neuropathic pain.

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